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ISBN

978-90-367-5435-4 (printed version)
978-90-367-5436-1 (digital version)

Printing

Printed by IMGRAFIA, ul. Piłkarska 44, 94-121 Łódź, Poland

Cover

Cover designed by Małgorzata Miller

RIJKSUNIVERSITEIT GRONINGEN

Mapping The Interactions Between *Staphylococcus aureus* And Host Immune Cells

Proefschrift

ter verkrijging van het doctoraat in de

Medische Wetenschappen

aan de Rijksuniversiteit Groningen

op gezag van de

Rector Magnificus, dr. E. Sterken,

in het openbaar te verdedigen op

woensdag 23 mei 2012

om 16:15 uur

door

Małgorzata Miller

geboren op 7 september 1981

te Łódź, Polen

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The Germ

A mighty creature is the germ,

Though smaller than the pachyderm.

His customary dwelling place

Is deep within the human race.

His childish pride he often pleases

By giving people strange diseases.

Do you, my poppet, feel infirm?

You probably contain a germ.

- Ogden Nash

CONTENTS

Chapter 1

Introduction and scope of this thesis

Introduction

Staphylococcus aureus (*S. aureus*) is a robust, non-motile, spherical bacterium that has emerged over the past century as a major threat for human health and well-being, causing infection outbreaks world-wide. Currently it is estimated that approximately one-third of the human population is colonized with *S. aureus*. While the carriage of this Gram-positive bacterium is of no serious consequence to most individuals*, S. aureus* has the potential to cause a wide range of diseases ranging from minor skin infections to more serious invasive diseases, such as bacteremia, sepsis, abscesses in various organs, osteomyelitis, endocarditis, pneumonia, and meningitis [2,3,4]. This pathogen is in fact capable of infecting nearly every organ and tissue in the body due to its ability to produce an arsenal of toxins, host-binding proteins and other virulence factors (Fig.1).

Virulence factors

The staphylococcal cell envelope

S. aureus is a Gram-positive bacterium that has a single cell membrane, which is coated with a thick cell wall composed of peptidoglycan (PepG) and various other polymers (Fig.1B). The cell wall is a meshwork of sugars (glycans) and proteins (peptides), which constitute about 50 percent of the staphylococcal cell. Compared to Gram-negative bacteria, *S. aureus* has an exceptionally thick and highly cross-linked layer of peptidoglycan. The peptidoglycan of Escherichia coli for example is 1.5-15nm thick, whereas the staphylococcal peptidoglycan layer reaches a thickness of about 30nm [5]. This thick peptidoglycan layer gives the bacterium shape and protects it from external unfavourable conditions and mechanical damage. Peptidoglycan fragments are released upon regulated cell wall turnover or bacterial cell lysis, thereby playing an important role in sepsis, shock and inflammation caused by *S. aureus* [6,7,8,9]. In addition, several *S. aureus* strains also produce a thin polysaccharide capsule in vivo to resist phagocytosis. Eleven capsule types have been identified, the most common being serotypes 5 (CP5) and 8 (CP8), each encoded by 16 gene operons on the bacterial chromosome [10]. Types 5 and 8 share similar sugar structures, and are composed of O-acetyl-β-D-N-acetylmannosaminuronic acid and N-acetylfucosamine [11]. Staphylococci expressing capsule are more virulent and persistent in animal infection models [12]. In *S. aureus*, the peptidoglycan is

buried within a complex cell surface matrix consisting of different molecules [5,13], such as surface proteins, capsular polysaccharides, usually required for the ability of different bacteria to cause disease, and teichoic acids (TAs) [12,14,15,16]. Teichoic acids are glycopolymers that play a crucial role in environmental stress, regulation of bacterial cell division and bacterial survival under disadvantageous conditions. The teichoic acids are usually constitutively produced and are either connected to peptidoglycan (wall teichoic acids, WTA) or to the cytoplasmic membrane (lipoteichoic acids, LTA).

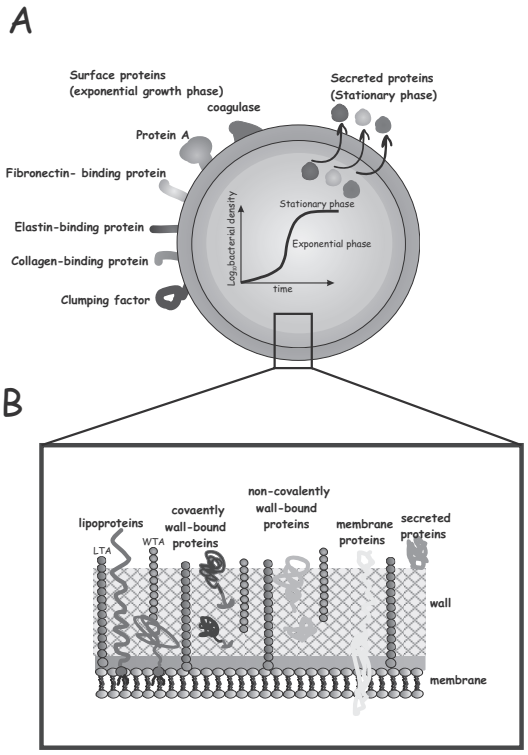

Figure 1. Schematic representation of staphylococcal virulence factors and their subcellular or extracellular localization.

Staphylococcal teichoic acids exhibit zwitterionic properties due to the presence of negatively charged phosphate groups and additional D-alanine residues on the repeating units, which have free positively charged amino groups [17,18,19,20]. The actual function of teichoic acids and the reasons why most Gram-positive bacteria produce them are not completely understood. WTA has been shown to be dispensable for the growth and survival of *S. aureus* and

Bacillus subtilis under laboratory conditions [21,22], but it does play an important role in host colonization and infection in vivo [23,24,25,26]. Moreover, teichoic acids are involved in a) protection against harmful molecules and environmental stresses, b) control of enzyme activities and cation levels in the cell envelope, and c) binding to receptors and surfaces [27]. Although purified WTAs of *S. aureus* are not very inflammatory, it has been reported that WTA can be associated with reduced bacterial recognition by the peptidoglycan recognition receptor (PGRP) of *Drosophila.* Therefore, WTA may be part of a general mechanism used by *S. aureus* and other Gram-positive bacteria, which limits the access of innate receptors to peptidoglycan, thereby enabling bacteria to evade detection and establish infection [28,29]. The staphylococcal LTA contributes to sepsis [30] and provokes the secretion of cytokines and chemoattractants (TNF-α, IL-1β, IL-10, IL-12, IL-8, leukotriene B4, complement factor 5a, MCP-1, MIP-1α and granulocyte colony-stimulating factor) by monocytes and macrophages [31,32,33,34,35,36].

In addition to teichoic acids, the staphylococcal cell envelope contains membrane proteins, lipoproteins (Lpp), covalently bound cell wall proteins and non-covalently bound cell wall proteins (Fig.1). Proteins anchored in the cell membrane and proteins covalently linked to the peptidoglycan can be cell surface-exposed. They are expressed during the exponential growth phase of *S. aureus* and are involved in host colonization, the first step in pathogenesis. Surface proteins specifically bind the host's extracellular matrix (ECM) and therefore are often called **MSCRAMMs** (microbial surface components recognizing adhesive matrix molecules). The best characterized representatives of the MSCRAMMs include fibronectin-binding proteins (FnbA, FnbB), and clumping factors (ClfA and ClfB), which can bind to integrins on the surface of host cells via fibrinogen or fibronectin bridges [37]. Another surface protein, Cna, serves as a collagen adhesin and is believed to facilitate invasive infection, particularly osteomyelitis [38]. Staphylococcal surface proteins also allow this bacterium to adhere to implanted medical devices covered in serum protein deposits, as well as intact epithelium. For example, the extracellular fibrinogen-binding protein (Efb) in addition to fibrinogen can also bind the free complement factor C3b [39].

Avoidance of Host Defences

When the outer physical barriers of the body (i.e. the skin or the mucosa) are breached by *S. aureus*, the bacteria are seriously confronted by the host's immune

system, comprising both innate and acquired responses. *S. aureus* infections stimulate strong inflammatory responses, involving the migration of neutrophils and macrophages to the site of infection. These cells engulf and dispose of the invading microorganism with help of antibodies and complement. In order to combat the host's immune system, *S. aureus* expresses proteins that inactivate components of the immune system through appropriate enzymatic activities or binding. The **Surface protein A** (Spa) is a cell wall-associated protein present in the large majority of *S. aureus* strains. It binds to the Fc region of immunoglobulin G and interacts with some Fab fragments [40,41]. A consequence of these interactions is the coating of the bacterial cell with IgG molecules that are in the incorrect orientation for recognition by the neutrophil Fc receptor. This could explain the antiphagocytic effect of protein A and its prominent role in the pathogenesis of *S. aureus.* Protein A-deficient mutants of *S. aureus* are phagocytosed more efficiently by neutrophils *in vitro* and show decreased virulence in several animal infection models [42,43]. The Fc-binding ability of Spa however is not the only mechanism to evade the immune system. Spa is also known to interfere with the activation of the classical pathway of the complement system [44]. In addition to Spa, almost all *S. aureus* strains express the **Sbi protein**. Sbi is 436-residue protein that is also found in the extracellular milieu. It consists of four globular domains, namely the immunoglobulin-binding domains I and II that bind to the Fc region of IgG and β_2-glycoprotein I [45,46,47], and the domains III and IV that were shown to bind to complement component C3 via its extracellular globular domains III and IV [48]. *S. aureus* also has the ability to inactivate the complement factor C3b and IgG molecules that are bound to the surface of opsonized bacterial cells. **Staphylokinase** (SAK) is a protein encoded by certain prophages that was shown to activate plasminogen (PLG) into plasmin (PL). Plasmin is a potent serine protease known for its capacity to degrade fibrin clots, extracellular matrix components and human IgG. Thus, incubation of PLG with SAK results in removal of anti-staphylococcal IgGs and C3b from the bacterial surface, which makes staphylokinase a unique anti-opsonic molecule [49]. Another protein that, similarly to staphylokinase, is secreted and acts on the surface of the bacterium is the **staphylococcal complement inhibitor** (SCIN). SCIN binds specifically to two C3 convertases (C4b2a and C3bBb), which interferes with the processing of C3 into microbe-bound C3b. SCIN can therefore block the phagocytosis and killing of *S. aureus* cells by human neutrophils [50,51]. On top of

that, about 60% of the *S. aureus* strains secrete the **chemotaxis inhibitory protein of staphylococci** (CHIPS), which blocks neutrophil chemotaxis by binding to the formylated peptide receptor and the C5a receptor on neutrophils [52,53,54]. Furthermore, *S. aureus* secretes the **Extracellular fibrinogen binding molecule** (Efb) that binds to fibrinogen and interferes with platelet aggregation [55,56,57]. Moreover, Efb also inhibits both the classical and alternative pathways of complement activation protecting *S. aureus* from complement-mediated opsonophagocytosis [39,58].

Intracellular survival of *S. aureus*

Although *S. aureus* is generally considered as an extracellular pathogen, it has been showed that it can invade a variety of non-professional phagocytes, such as human endothelial cells, bovine epithelial cells and murine fibroblasts. Chronic infections like osteomyelitis, the frequent failure of antibiotic treatments to overcome staphylococcal infections, or their recurrence might be related to the ability of *S. aureus* to invade and persist within certain types of host cells, providing a protected niche [59,60,61]. Survival of *S. aureus* within the host macrophage requires the bacterium to be resistant to the normal bactericidal mechanisms. To survive phagocytosis, one of the primary defense mechanisms of the innate immune system, *S. aureus* is equipped with catalase (KatA), thioredoxin (TrxA), superoxide dismutases (SodA and SodM) and glutathione peroxidase (GpxA), among others, to combat any reactive oxygen species they may encounter [62]. It has been reported that staphylococcal catalase and superoxide dismutase (SOD) combat reactive oxygen species enabling *S. aureus* to persist within macrophages, inducing local inflammation, and causing an increased induction of serum TNF–α and IL-6 [63]. Furthermore *S. aureus* redirects from the endosomal pathway to autophagosomes. In this way *S. aureus*-induced autophagy is required for staphylococcal replication, subsequent escape from autophagosomes into the cytoplasm, and eventually *S. aureus*-induced host cell death [64]. Moreover, it is now known that professional phagocytes provide a beneficial environment for intracellular survival of *S. aureus*. Consistent with this notion, recent studies have revealed that *S. aureus* is highly resistant to neutrophil- and macrophage-mediated killing [65,66].

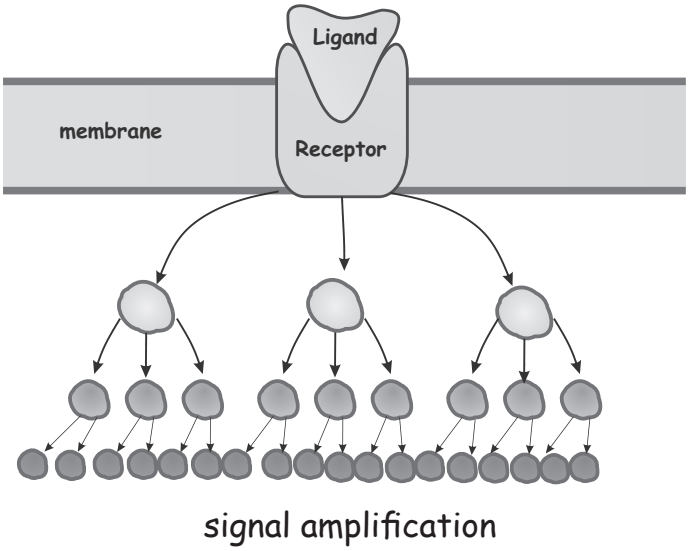

Figure 2. Schematic representation of a signal transduction pathway. Upon stimulation through, for example ligand binding or chemical modification, a signal is generated by an appropriate receptor. Through protein-protein interactions and phosphorylation reactions involving activated proteins, the original signal is amplified and transduced to response regulators that orchestrate the cellular responses to the stimulus that was received by the receptor.

Inflammatory signaling during *S. aureus* infection

The ability of both bacterial and eukaryotic cells to adapt their behavior to changes in their surrounding environment is crucial if they exist in dynamic and challenging environments. Furthermore, cells must effectively communicate with one another, especially when cooperative interactions are needed. In order to detect a signal and translate this signal into a cellular event, highly specialized mechanisms are used by the cells. These mechanisms are collectively known as signal transduction. Signal transduction is achieved through specific classes of proteins and the interactions between them. For example, when a cell of the immune system detects bacterial compounds in its environment, it reacts to the signal by activating signal transduction cascades to respond to a potential invasion. As a result immune cells can release cytokines (to communicate with other cells), change morphology (to become more motile) or alter their gene expression.

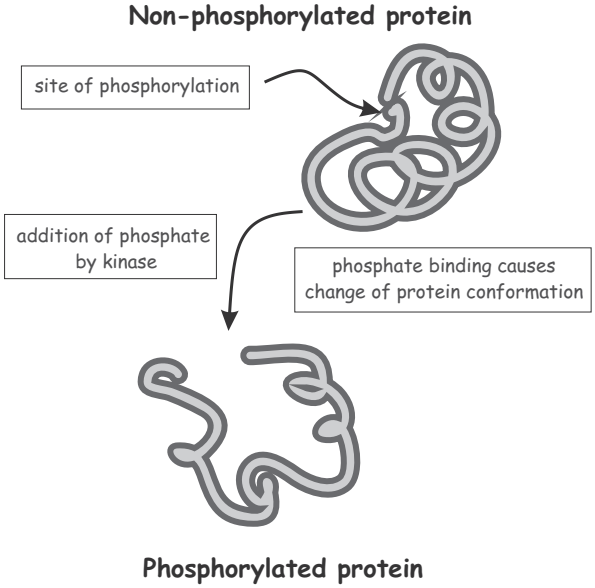

Figure 3. Phosphorylation and dephosphorylation are key mechanisms in the transduction of signals within cells. When a protein kinase becomes phosphorylated it can catalyze subsequent phosphorylation steps, thereby transducing a signal.

One of the key regulatory mechanisms underlying signal transduction is protein phosphorylation. The transfer of phosphate to proteins is performed by kinases, and the dephosphorylation by phosphatases (Figure 3). The phosphorylation usually results in the activation of a protein, which then is able to activate one or more other proteins. Errors in signal transduction can lead to misinterpretation of the information by cells, which subsequently can results in, for example, the onset of cancer or improper inflammatory responses during infections. Although the signaling pathway itself is linear, it still remains a major challenge to understand the extensive cross talk that exists between different signal transduction pathways. Currently available micro-array technologies analyze nucleotides (DNA and RNA), however these methods are not yet suitable for deciphering the complex dynamics of cell signaling reactions**.**

The innate immune system constitutes the first line of host defenses during infection and therefore it plays a crucial role in the early recognition and subsequent triggering of a pro-inflammatory response to invading pathogens. The adaptive immune system, on the other hand, is responsible for the elimination of pathogens in the late phases of infection and in the generation of immunological memory. Whereas the adaptive immune response is characterized by specificity

developed by clonal gene rearrangements from a broad repertoire of antigen-specific receptors on lymphocytes, the innate immune response is mediated primarily by phagocytic cells and antigen-presenting cells (APCs), such as granulocytes, macrophages, and dendritic cells (DCs), and has been regarded as relatively nonspecific. The innate immune response relies on the recognition of evolutionarily conserved structures on pathogens, termed **pathogen-associated molecular patterns (PAMPs)**, by a limited number of germ line-encoded **pattern recognition receptors (PRRs)** of which the family of **Toll-like receptors** (TLRs) has been studied most extensively. In humans, 11 Toll-like receptors (TLRs) have so far been identified [67,68].

Toll-like receptor 2 (TLR-2) has been reported to have a broad role as a pattern recognition receptor for a variety of microbes and microbial structures. These include lipoproteins from pathogens, such as mycobacteria, spirochetes, and mycoplasmas, lipoarabinomannan from mycobacteria, the glycosylphosphatidylinositol anchor from *Trypanosoma cruzi*,and zymosan from fungi [67]. Furthermore, TLR2 has been implicated in the recognition of staphylococcal peptidoglycan and lipoteichoic acid (LTA) [69,70,71,72,73,74]. TLR2 thus seems to detect various specific components of pathogens, but it is presently not clear how one receptor like TLR2 can recognize such a wide spectrum of stimuli. It is believed that other receptors, such as CD14, TLR1, or TLR6 are also involved in LTA recognition by cooperation with TLR-2 [75,76,77,78,79,80]. Furthermore, TLR1 and TLR6 have been shown to mediate the discriminatory recognition of triacyl and diacyl lipopeptides by TLR2. TLR6 is necessary for TLR2 to detect MALP2 (macrophage-activating lipopeptide 2) from *Mycoplasma pneumoniae*, which is only diacylated [79]. In contrast, TLR1 is necessary for the response to triacyl lipopeptides and mycobacterial lipoproteins [80,81]. Since staphylococci also produce a set of lipoproteins and LTA, it is possible that these lipid-modified compounds are indeed detected by TLR2 and that their either diacylated or triacylated states dictate the contributions of TLR1 or TLR6 to their recognition [82]. Another receptor that cooperates with TLR-2 is CD14. CD14 is a key co-receptor expressed on the surface of macrophages and polymorphonuclear leukocytes (PMNs). For example, CD14 is necessary for full induction of the inflammatory response by LPS-stimulated TLR-4 [83]. Several studies have demonstrated that different components of *S. aureus*, such as peptidoglycan and LTA also interact with the CD14 molecule [32,33,84]. Thus, it has been suggested

that CD14 is a functional receptor for staphylococcal peptidoglycan and LTA. Interaction with TLR-2 has also been reported for the CD36 receptor, and coexpression of either CD36 or TLR-6 with TLR-2 significantly increases the TLR-2 responses to LTA.

TLR-2 is an essential receptor for the recognition of *S. aureus.* However it remains interesting to find out why macrophages from TLR-2-deficient mice stimulated with *S. aureus* still produce pro-inflammatory mediators [85]. This suggests that host cells must have some alternative receptors that are involved in *S. aureus* recognition. TLRs are involved in recognition of pathogens in the extracellular compartment, whereas Nod receptors are involved in intracellular sensing of microorganisms and their products [86]. The **Nod receptors** (Nod1 and Nod2) are intracellular pattern-recognition molecules of the Nod-like receptor (NLR) family. Nod1 is ubiquitously expressed, while Nod2 expression is restricted to monocytes, macrophages, dendritic cells and intestinal Peneth cells. Both proteins are implicated in the detection of bacterial peptidoglycan. Nod1 senses peptidoglycan fragments containing meso-diaminopimelic acid and Nod2 seems to be a general sensor, which is activated by muramyl dipeptide (MDP) – the common motif of peptidoglycan from Gram-positive and Gram-negative bacteria. Upon activation, Nod1 and Nod2 induce I kappa B kinase (IKK) and NF-κB, which results in pro-inflammatory responses. Although it was initially thought that *S. aureus* is an extracellular bacterium, several observations indicate that *S. aureus* peptidoglycan is recognized by cytoplasmic Nod2. Recent studies have shown that *Nod2*-deficient mice are more susceptible to *S. aureus*, which results in defective neutrophil phagocytosis and a higher bacterial tissue burden [87]. While Nod2 and TLR2 induce immune responses via the activation of the transcription factor NF-κB and MAP kinases, the group of NLRs that include Nlrp3 and Nlrc4 are critical for the **inflammasome** activation and IL-1β secretion in response to bacterial and endogenous stimuli [88]. Upon microbial stimulation, the IL-1β precursor is induced in macrophages via NF-κB activation. Subsequently, pro-IL-1β is processed into mature IL-1β by the enzymatically active heterodimer composed of a 10- and a 20-kDa chain of caspase-1. Nlrp3 is essential for caspase-1 activation in response to a variety of microbial molecules in combination with ATP and particulate matter including ureate crystals, silica particles and aluminum salts [89,90,91]. Recent studies showed that the adaptor protein Asc and IL-1β can play critical roles in the neutrophil recruitment in staphylococcal skin infections and

the elimination of *S. aureus in vivo* [92,93]. Furthermore, infection of macrophages with *S. aureus* induced caspase-1 activation via Nlrp3 *in vitro*, and the combination of staphylococcal hemolysins and lipoproteins plays a critical role in the Nlrp3 inflammasome activation [94,95].

Another group of receptors involved in *S. aureus* recognition are the **peptidoglycan recognition proteins (PGRPs).** Four PGRPs are present in humans, PGLYRP1, PGLYRP3, PGLYRP4 and the PGLYRP3:PGLYRP4 heterodimer. Each of them contains at least three conserved peptidoglycan-binding domains. Peptidoglycan can be detected in different cellular compartments depending on the type of PGRP, which can be membrane bound, stored in vesicles, or secreted into the extracellular milieu [96]. Mammalian PGLYRP2 is an N-acetylmuramoyl-L-alanine amidase that hydrolyzes the lactyl bond between the MurNAc and L-Ala in bacterial peptidoglycan. The preferred substrates for recognition are soluble peptidoglycan fragments, such as products of peptidoglycan digestion by peptidoglycan-hydrolyzing enzymes like lysozyme or other hydrolases. By contrast, intact cross-linked peptidoglycan in the bacterial cell wall is a poor PGLYRP2 substrate. Notably, due to as yet unidentified activities, PGLYRP1, PGLYRP3, PGLYRP4, and PGLYRP3:PGLYRP4 have bactericidal or bacteriostatic properties for many pathogenic and non-pathogenic Gram-positive and Gram-negative bacteria [97].

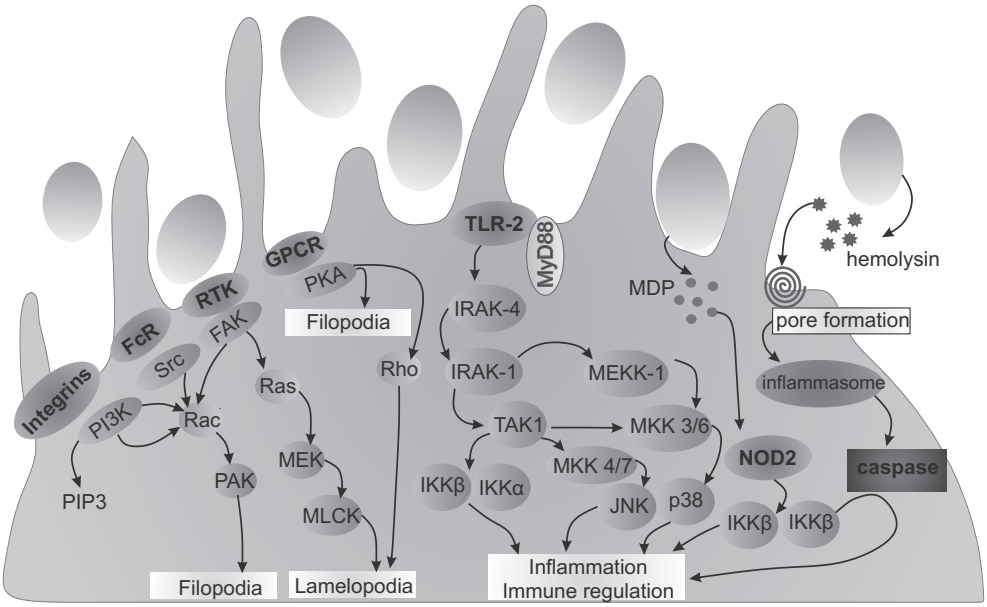

Figure 4. Signal transduction in macrophages during *S. aureus* infection.

The staphylococcal eukaryotic-like serine/threonine kinase PknB

Serine/threonine kinases and phosphatases are well known for their involvement in eukaryotic signaling cascades. These kinases specifically phosphorylate serine or threonine residues in their substrates. Interestingly, while this was initially overlooked, it has become clear in recent years that also in prokaryotes serine/threonine kinases can play important roles in regulatory mechanisms. The prokaryotic signal transduction pathways most often involve so-called two-component regulatory systems, which are composed of a histidine kinase and a response regulator. In addition, eukaryotic-like serine/threonine protein kinases (ESTKs) are employed by many prokaryotes for the regulation of various cellular functions, such as stress responses, biofilm formation, sporulation, metabolism and developmental processes. The first ESTK was identified in *Myxococcus xanthus* in 1991 [67]. Since then, similar ESTKs and ESTPs have been identified and characterized in both Gram-negative and Gram-positive pathogens, including *Salmonella enterica*, *Pseudomonas aeruginosa*, *Streptococcoccus agalactiae*,

Streptococcus pneumoniae, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Bacillus subtilis* and *Streptococcus mutans*, as well as *Mycobacterium tuberculosis* [98,99,100,101,102]. Structurally, ESTKs vary across organisms depending on their function(s). The Gram-positive bacterial ESTKs are usually membrane-bound with an extracellular sensing PASTA domain (i.e. a penicillin-binding protein and serine/threonine kinase-associated domain), a variable number of trans-membrane domains (often just one), and a catalytic, intracellular kinase domain (Fig. 5). In this way, a bacterium can transmit an appropriate signal across its cell membrane.

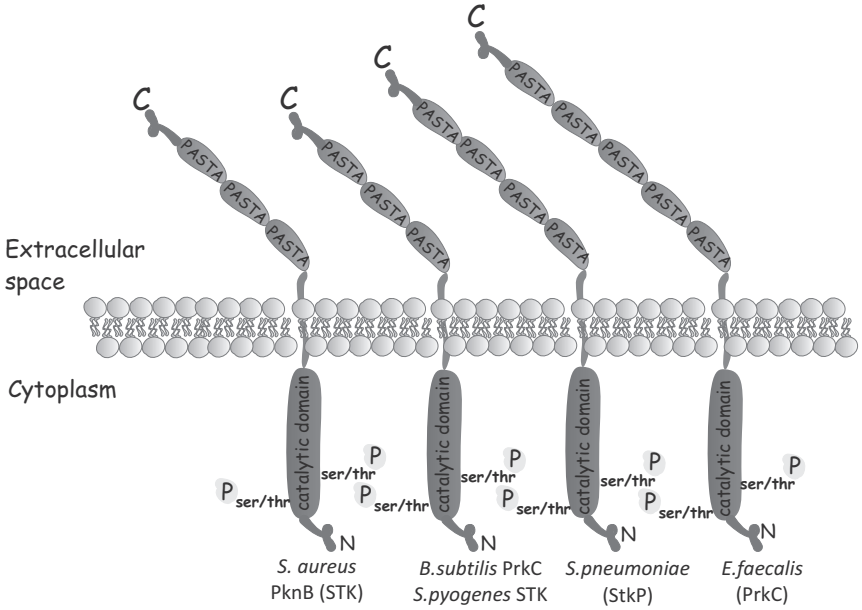

Figure 5. Staphylococcal eukaryotic-like serine/threonine kinases, including PknB of *S. aureus*.

The staphylococcal STPK, often referred to as PknB, is composed of three extracellular PASTA domains (penicillin-binding domains), a central transmembrane domain and an intracellular kinase domain [103]. It was reported that PknB of *S. aureus* is involved in the regulation of various metabolic pathways and cell wall synthesis [102,104]. Importantly, PknB regulates autolysins, which are peptidoglycan hydrolases that play important roles in bacterial cell wall turnover, cell division and cell separation. Since peptidoglycan is needed to maintain bacterial cell integrity and viability, it is of crucial importance to maintain a proper

balance between new synthesis of peptidoglycan and its degradation. It was recently reported that *pknB* mutant *S. aureus* cells have a 2.2-fold decreased expression of the major *S. aureus* autolysin gene *atl* and a 2.3- to 2.8-fold induction of two autolysis regulators (*fmtA* and *lytR)* and two murein hydrolase gene regulators (*lrgA* and *lrgB*) [103]. Furthermore *pknB* mutant *S. aureus* strains produce less UDP-N-acetylmuramates, which are the main precursors for peptidoglycan synthesis and they are more resistant to Triton X-100-induced autolysis [103,105]. Moreover, PknB was shown to determine the infectivity of *S. aureus* in a mouse kidney abscess model [106].

Scope of this thesis

The main objectives of the research described in this thesis were to elucidate the responses of human and mouse macrophages towards *S. aureus* infection, to pinpoint determinants of *S. aureus* that have key roles in these host cell responses, and to map the responses of *S. aureus* to macrophages. Moreover, despite the significance of eukaryotic-like Ser/Thr kinases in bacterial pathogenesis, nothing was known about the roles of the staphylococcal PknB in the onset of host cell responses toward *S. aureus*. Therefore, the present studies were for a large part focused on the exploration of possible roles of PknB in staphylococcal infections.

Chapter 2 describes the complex phosphorylation networks that are triggered in human macrophages encountering staphylococcal cells. Moreover, the results challenge the classical concept that macrophage responses are mainly mediated through Toll-like receptor 2 and NF-κB signaling and highlight the important role of the stress-activated MAP kinase signaling in the host defense. In addition, gel-free proteomics was employed to monitor staphylococcal responses to the THP-1 macrophages.

The studies described in **Chapter 3** show that full-size soluble PknB is present in the medium of growing *S. aureus* cells. Peptide microarrays with known human phosphorylation sites and mass spectrometry revealed that PknB is a proline-directed kinase, which can phosphorylate specific human targets. The observed target specificity of PknB indicates possible roles for this enzyme in a wide range of host cell signalling processes during *S. aureus* infection.

Chapter 4 studies the roles of *S. aureus* PknB in the infection of human immune cells using enzyme-linked immune sorbent assays (ELISA) and kinase profiling. This chapter pinpoints the crucial role of PknB in the onset of inflammatory host cell responses. Importantly, the results imply that *S. aureus* PknB could be a well-suited target for combating staphylococcal infections, especially through the minimization of inflammatory responses.

In **Chapter 5** the usefulness of a penicilinase translocation assay to screen different *S. aureus* secretion mutants for intracellular protein secretion and survival in mouse macrophages was explored. The results show that the assay can indeed be used for this purpose but, unfortunately, it does not allow a distinction

between effects of particular mutations on intracellular protein secretion and survival.

This thesis ends with **Chapter 6**, which summarizes and discusses the research described and provides an outlook on relevant possibilities for future research on the interactions between *S. aureus* and human immune cells.

Chapter 2

Mapping of interactions between human macrophages and *Staphylococcus* aureus reveals an involvement of MAP kinase signalling in the host defence

J. Proteome Res., **2011**, *10* (9), pp 4018–4032

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Abstract

Staphylococcus aureus is a dangerous opportunistic human pathogen that causes serious invasive diseases when it reaches the blood stream. Recent studies have shown that *S. aureus* is highly resistant to killing by professional phagocytes, and that such cells even provide a favourable environment for intracellular survival of *S. aureus*. Importantly, the reciprocal interactions between phagocytes and *S. aureus* have remained largely elusive. Here we have employed kinase profiling to define the nature and time resolution of the human THP-1 macrophage response towards *S. aureus*, and proteomics to identify the response of *S. aureus* towards macrophages. The results of these studies reveal major macrophage signalling pathways triggered by *S. aureus*, and proteomic signatures of the responses of *S. aureus* to macrophages. We also identify human proteins bound to *S. aureus* that have potential roles in bacterial killing and internalization. Most noticeably, our observations challenge the classical concept that macrophage responses are mainly mediated through Toll-like receptor 2 and NF-κB signalling, and highlight the important role of the stress-activated MAP kinase signalling in orchestrating the host defence.

Introduction

Professional phagocytes play an essential role in the host defence against bacterial pathogens by recognizing, engulfing, and eventually eradicating invading bacteria. Phagocytosis is the process by which cells engulf large particles through the formation of pseudopodia or invaginations of the plasma membrane. In order to discriminate between 'self' and 'non-self' agents mammalian professional phagocytes, such as neutrophils, dendritic cells and macrophages display a number of phagocytic receptors [107,108]. Furthermore, the interaction between pathogen and macrophage can be promoted either by opsonins, such as complement proteins and specific antibodies that coat the bacterial cell surface (*i.e.* opsonic phagocytosis), or by direct binding to surface receptors (*i.e.* non-opsonic phagocytosis). The ability of macrophages to directly interact with non-opsonized bacteria is based on the recognition of conserved Pathogen-Associated Molecular Patterns (PAMPS) by pattern recognition receptors (PRRs) [107], most prominently the Toll-like receptors (TLRs). Activation of NF-κB through TLR activation constitutes a canonical cellular host defence response to pathogen activation, but also many other pathways are activated. Importantly, the relative contributions of the various pathways in the cellular response to pathogen exposure are so far poorly understood.

Most bacterial structures are recognized by more than one receptor and these receptors can also interact with each other. Thus, the activation of various sets of receptors will lead to the activation of a complex network of signalling pathways due to cross-talk between the activated receptors and various downstream signalling molecules. Integration of these signalling events triggers cytoskeletal rearrangements associated with cell surface modifications that lead to engulfment of the bound bacteria. After receptor engagement and engulfment, the bacteria will be internalized and enclosed in a phagosome. Newly formed phagosomes are not yet adjusted for killing and degradation of internalized bacteria. To acquire these properties, phagosomes fuse with endosomes and eventually with lysosomes. The resulting phagolysosome contains a membrane-associated NADPH-oxidase, which causes the formation of reactive oxygen species that effectively eradicate internalised bacteria through a mechanism named the oxidative burst. Moreover, the phagolysosomes contain a cocktail of degradative enzymes (*e.g.* lipases, proteases, nucleases), which serve to eliminate internalized pathogens.

Despite the presence of multiple defence systems, various pathogens can survive inside human host cells with the support of evasive mechanisms. Some bacterial species interfere with the ability of phagocytes to engulf them, either by inhibiting or degrading opsonic antibodies or complement [50], or by directly impairing the phagocytic process in macrophages and neutrophils [109]. These different strategies are exemplified by the mechanism used by *Mycobacterium tuberculosis, Listeria monocytogenes* and *Legionella pneumophila. M. tuberculosis* uses a number of effector molecules, including lipids (phosphatidylinositol mannoside [PIM] and lipoarabinomannan [LAP]), phosphatidylinositol-3-phosphate (PI(3)P), and the phosphatase SapM to arrest phagosome maturation at an early stage and to escape from the phagosome [110]. *L. monocytogenes*, the causative agent of listeriosis, escapes the phagosome through secretion of listeriolysin O and the phospholipases PlcA and PlcB [111]. Having reached the cytoplasm, *L. monocytogenes* replicates and becomes motile by using actin 'comet tails' generated by the effector ActA [112]. *Legionella pneumophila* causes legionellosis or Legionnaires' disease. Upon phagocytosis, this bacterium impairs fusion with endolysosomal compartments and instead promotes fusion with endoplasmic reticulum (ER)-derived membranes [113].

Staphylococcus aureus is a dangerous opportunistic human pathogen, carried by about 30% of the population. It has been implicated in a variety of diseases, ranging from minor skin infections to more serious invasive diseases. A large number of virulence factors are known to contribute to staphylococcocal pathogenesis. These include surface proteins assisting in host colonization [114], invasins and proteases that promote bacterial spreading, surface factors such as protein A that inhibits phagocytic engulfment, and toxins that damage host cell membranes [115]. These multiple virulence mechanisms *plus* various antibiotic resistance strategies have made *S. aureus* exceptionally successful in subverting its human host. Notably, for many years *S. aureus* was considered to be an extracellular pathogen. Recent studies have, however, shown that this organism has also the capacity to function as an intracellular pathogen [62,65,66]. It has been reported that *S. aureus* can survive in endothelial and epithelial cells [59] as well as in osteoblasts [60] thereby causing persistent infections such as endocarditis [61], and osteomyelitis . Moreover, it is now known that professional phagocytes provide a favourable environment for intracellular survival of *S.*

aureus. Consistent with this notion, recent studies have revealed that *S. aureus* is highly resistant to neutrophil- and macrophage-mediated killing [65,66].

Defining the mechanisms by which *S. aureus* successfully colonizes and subverts its human host remains highly complicated. A dissection of host signalling pathways involved in staphylococcal infections is even more troublesome without prior knowledge as to which pathways are involved. Kinase profiling analyses implement array technologies that comprehensively measure enzymatic signalling activities (*i.e.* kinase activities) present in whole cell lysates, usually by employing peptide substrates [116]. For this purpose, peptide arrays have been assembled that contain the entire complement of consensus phosphorylation sites of protein kinases deposited in the Phosphobase (http://phospho.elm.eu.org/). These arrays allow the detection of a very wide range of phosphorylation events, even those mediated by unconventional prokaryotic kinases [117], and they have been used to provide comprehensive descriptions of complex kinase activities present in whole cell lysates [118,119]. Here we have employed kinase profiling to approach questions involving the nature and time resolution of the cellular human THP-1 macrophage response towards *S. aureus* infection. In parallel, gel-free proteomics was employed for monitoring staphylococcal responses to the THP-1 macrophages. Interestingly, our results challenge the classical concept that macrophage responses to bacteria like *S. aureus* are mainly mediated through TLR and NF-κB signalling, and they highlight the important role of the stress-activated MAP kinase signalling cassettes in orchestrating the host defence.

Results and Discussion

Signal transduction in THP-1 macrophages upon interaction with *S. aureus*

Phagocytosis of a bacterial pathogen is a complicated process that involves numerous interactions not only between the pathogen and the phagocyte, but also between signalling pathways within the phagocyte. Eventually, these interactions lead to pathogen internalization and destruction. However, bacteria such as *S. aureus* have evolved many effective mechanisms for immune evasion, and it seems likely that phagocyte invasion by these bacteria might be an important step in the invasion of host cells and subsequent intracellular survival. To obtain insights in the changes in signal transduction in human macrophages

upon phagocytosis of *S. aureus*, we incubated human THP-1 macrophages for 15 min, 1 h or 24 h with *S. aureus* NCTC 8325 and analyzed the effects on kinase activity employing peptide arrays (schematically represented in Figure 1). The *S. aureus* strain NCTC 8325 was selected for these studies, because this strain has been used in many previous studies on staphylococcal virulence, and because its sequenced genome facilitated the proteomics analyses of staphylococcal cells exposed to the THP-1 macrophages (see below). As shown in Figure 2, *S. aureus* cells were effectively bound and internalized by THP-1 macrophages already after 15 min of incubation. Importantly, substantial numbers of the internalized *S. aureus* cells were able to survive for at least 24 h (Figure 2C). Kinase profiling of the responses within the macrophages revealed that 366 peptides are substrates of kinases of which the activities are significantly different upon incubation with or without *S. aureus*. We then grouped these peptides according to the signalling pathways in which the respective kinases are involved, and also by taking into account whether the respective kinase activity is up- or downregulated compared with the control of uninfected macrophages. Supplementary Table 1 lists the peptide substrates that were phosphorylated to significantly different extents when THP-1 macrophages were incubated with or without *S. aureus* for 15 min, and Supplementary Table 2 lists the peptide substrates that were phosphorylated to significantly different extents when THP-1 macrophages were incubated with or without *S. aureus* for 1 h or 24 h. Supplementary Tables 1 and 2 also provide information on the corresponding protein substrates and upstream kinases. Further details on the kinase profiling are presented in Supplementary Table 3. All the supplementary tables are available on the *Journal of Proteome Research* (JPR) website:
http://pubs.acs.org/doi/abs/10.1021/pr200224x?prevSearch=Miller%2BMalgorzata&searchHistoryKey=.

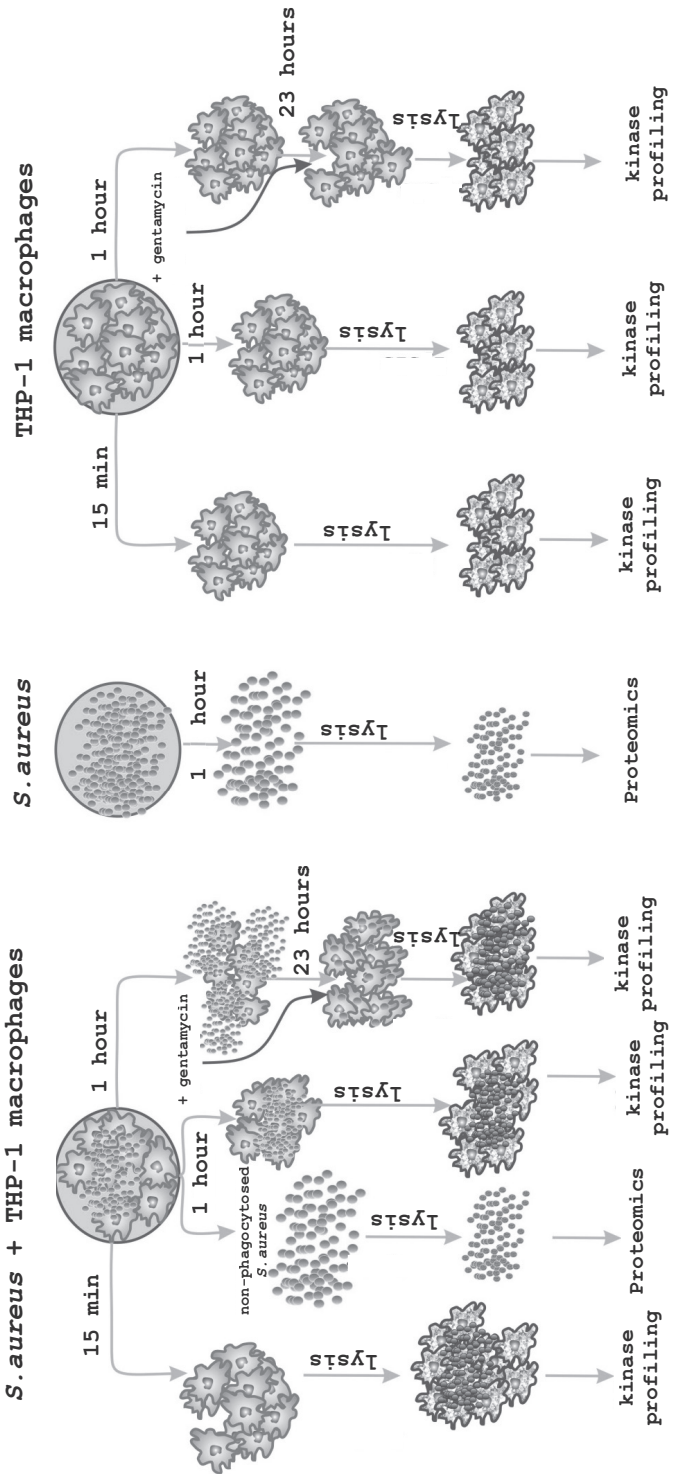

Figure 1. Schematic representation of work flows. The diagram gives an overview of the work flows to monitor the responses of THP-1 macrophages to *S. aureus* cells by kinase profiling, and (2) the responses of *S. aureus* to THP-1 macrophages by proteomics. For details see Materials and Methods.

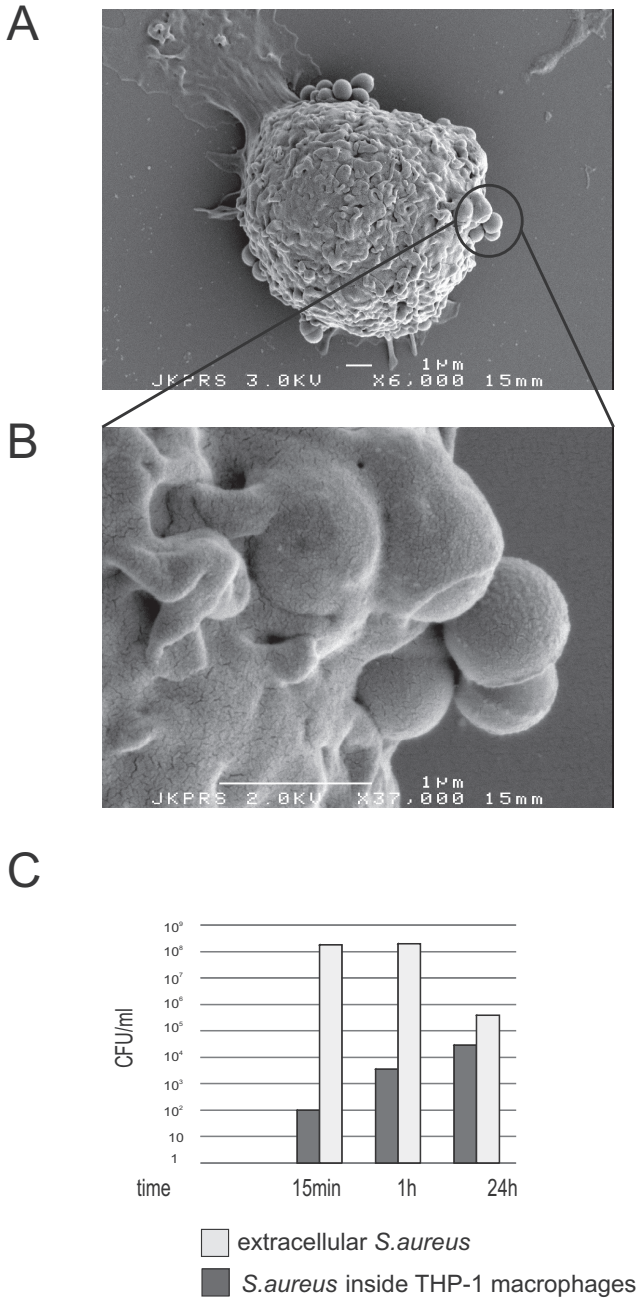

Figure 2. Scanning Electron Microscopic image of *S. aureus* phagocytosis by a THP-1 macrophage after 15 minutes of incubation. Panels A and B represent different magnifications of the same image. (C) Survival rates of *S. aureus* cells inside and outside the THP-1 macrophages. Colony forming units (CFU) were determined by plating on blood agar.

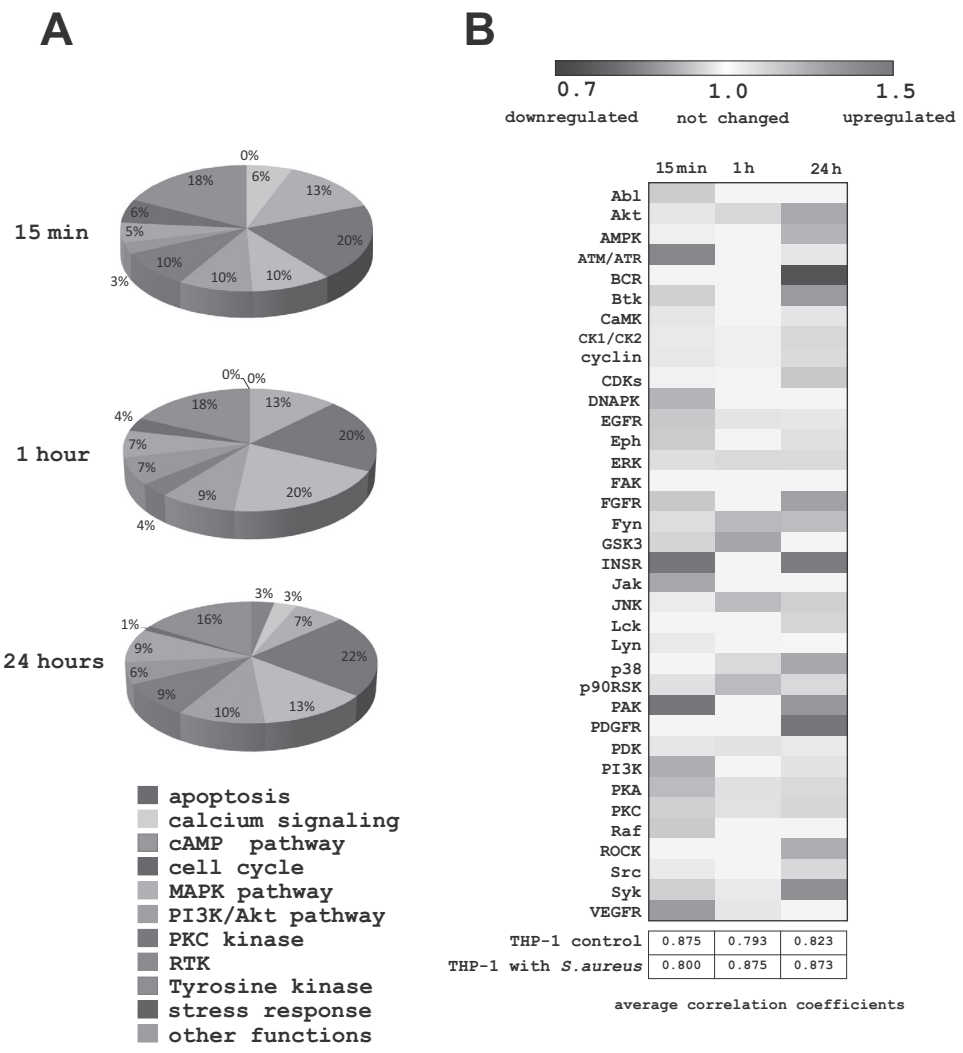

Figure 3. Activity of different kinases and phosphorylation pathways in THP-1 macrophages incubated with *S. aureus*. THP-1 macrophages were incubated with *S. aureus* NCTC 8325 cells for 15 min, 1 h or 24 h. As a control, macrophages were incubated without *S. aureus* cells. Kinase profiling and data analyses were performed as described in the Materials and Methods section. (A) Overview of kinases and signalling pathways that are influenced upon incubation of THP-1 cells with *S. aureus*. (B) Up- and downregulation of kinase activities in THP-1 macrophages incubated with *S. aureus*. A fold change of 1 represents the situation where the phosphorylation of respective peptides is identical in THP-cells incubated with or without *S. aureus*. A fold change of > 1 indicates that the phosphorylation intensity of particular peptides was increased when THP-cells were incubated with *S. aureus*, and a fold change <1 indicates that the phosphorylation intensity of particular peptides was decreased when THP-cells were incubated with *S. aureus*. Calculated values of the 36 presented up- or down-regulated kinase activities are based upon the data in Supplementary Table 3. The Table below the heat map lists the average correlation coefficients of three data sets obtained from samples of THP-1 macrophages incubated with *S. aureus* or the control samples of THP-1 macrophages incubated without *S. aureus* at each time point (15 min, 1 hour and 24 hours). The respective correlation coefficients were derived from the plots presented in Supplementary Figure 3, which is available on the Journal of Proteome Research website http://pubs.acs.org/doi/abs/ 10.1021/pr200224x?prevSearch=Miller%2BMalgorzata&searchHistoryKey=..

Early signalling events and regulation of actin dynamics

Incubation of THP-1 macrophages with *S. aureus* for 15 min resulted in the activation of known signalling pathways for early phagocytosis and cytoskeletal rearrangement. During the first 15 min post infection, we noted activation of the cyclic adenosine 3,5-monophosphate (cAMP)-dependent protein kinase A (PKA), Ca^{2+}-dependent kinases (*i.e.* the calmodulin-dependent kinase II (CaMKII) and the protein kinase C (PKC)), the phosphatidylinositol 3-kinase (PI3K), the Akt kinase, and the mitogen-activated protein kinase ERK. Moreover, we observed induction of the ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases, which are involved in the DNA damage response, and of tyrosine receptor kinases that regulate the insulin receptor (INSR) and the vascular endothelial growth factor receptor (VEGFR). An overview of the kinases that were found to be activated in THP-1 macrophages upon incubation with *S. aureus* is presented in Figure 3, and the respective signalling pathways are schematically represented in Figure 4.

The observed activation of PKA is most likely regulated by a change in the cellular cAMP concentration (Figure 4). The secondary messenger cAMP has a fundamental role in the cellular responses to many extracellular stimuli, and it controls a diverse range of cellular processes. The main source of cAMP in the cell is ATP, which is converted to cAMP by adenylyl cyclase (AC). Binding of PKA to cAMP results in a conformational change in PKA, which causes the dissociation of the holoenzyme and activation of PKA.

Interestingly, the presence of *S. aureus* results in activated CaMKII, which implies an upstream regulation by the phospholipase C (PLC) and changes in the cytoplasmic Ca^{2+} concentration as well as activation of the downstream Ca^{2+} signalling pathways (Figure 4). The cytoplasmic concentration of Ca^{2+} is regulated by PLC, which hydrolyses phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) in the plasma membrane. The inositol phosphates generated by PLC (*e.g*. IP3) bind to specific channels in the endoplasmic reticular (ER) membrane thereby causing Ca^{2+} release from the ER into the cytoplasm. This then results in an increase of the cytoplasmic Ca^{2+} concentration from ~100 nM to up to 1 µM. This rise in the cytoplasmic Ca^{2+} levels is known to trigger CR3- and FcγR-mediated phagocytosis by neutrophils and macrophages [120,121]. Moreover, Ca^{2+} regulates cytoskeletal changes [122], actin filament severing [123], and it binds to annexins and calmodulin (CaM) [124,125]. Binding of Ca^{2+} to CaM, triggers a

conformational change and allows the Ca^{2+}-CaM complex to bind to numerous effector proteins [126]. Ca^{2+}-CaM is required for fusion of endocytic vesicles with the yeast vacuole (a homologue of mammalian lysosomes), endosome-endosome fusion, receptor recycling, exocytosis, and transcytosis [127,128,129]. Ca^{2+}-CaM also binds to the calmodulin-dependent kinase II (CaMKII), which is a serine/threonine kinase that was shown to regulate endosome-endosome fusion [130,131]. In resting cells, CaMK is present in its auto-inhibited state. However, binding of Ca^{2+}-CaM to CaMKII relieves autoinhibition and activates CaMKII [132], which seems to happen when THP-1 cells are incubated with *S. aureus*.

The observed activation of PKC by the presence of *S. aureus* is consistent with the finding that CaMKII is activated (Figure 4). This relates to the fact that the second product of $PtdInsP_2$ hydrolysis by PLC is diacylglycerol DAG. The main function of DAG, which remains associated with the membrane, is activation of kinases of the PKC family. PKCs are cytosolic proteins, but it has been proposed that they might be associated also with the plasma membrane in response to various stimuli such as Ca^{2+} and/or DAG [133]. Notably, it was reported that PKCα localizes to the phagosome membrane during FcγR- and complement-mediated phagocytosis [134,135]. While complement was absent in the present studies due to heat inactivation, our media probably contained some fetal bovine IgGs. However, we believe that FcγR-mediated phagocytosis plays only a minor role, if any, in the present studies because of the activity of the IgG-binding proteins SpA and Sbi of *S. aureus*, which bind the Fc domain of IgG at the bacterial cell surface to evade recognition by FcγR.

Our kinase profiling results show also an activation of the PI3K pathway. PI3K phosphorylates phosphatidylinositol 4,5-biphosphate lipids ($PtdInsP_2$, PIP2), which are the major regulators of actin remodelling [136] (Figure 4). Accordingly, PI3K regulates phagocytosis by modulating pseudopod extension [137] and phagosome maturation [138]. Furthermore, PI3K activates many downstream signalling molecules, such as the phosphoinositide-dependent kinase-1 (PDK-1), anti-apoptotic Akt [139], PKC [140,141] and the Rho family GTPase protein Rac. Notably, Rac is a key regulator of cytoskeletal extensions, actin polymerization, particle engulfment and the formation of lamellipodia and phagosomes [142,143], which requires the Rac-mediated activation of the p21-activated kinase 1 (PAK1) [144]. Indeed, kinase profiling revealed the activation of PAK1, which implies that Rac was activated in the presence of *S. aureus*. It has been suggested that PAK is

an upstream regulator of the vimentin network [145] and, interestingly, this may also apply to *S. aureus* infections since we observe up to two-fold increased phosphorylation of the vimentin peptide by PAK. Vimentin is a member of the intermediate filament (IF) family of proteins and, together with microtubules and actin filaments, vimentin forms the cytoskeletal framework in the cytoplasm of various eukaryotic cells. The reorganization of IF is regulated by serine/threonine kinases through IF phosphorylation. Although it was shown that PAK phosphorylates vimentin only in smooth muscle cells, our results suggest that regulation of vimentin by PAK might play an important role in phagocytosis of *S. aureus*, which is a novel finding.

Also the anti-apoptotic ERK pathway is induced during the phagocytosis of *S. aureus* by human THP-1 macrophages. The phosphorylation profile indicates activation of R-Ras by receptor tyrosine kinase phosphorylation. Ras is an upstream regulator of the Raf-MEK-ERK1/2 cascade (Figure 4). Interestingly, we observed increased phosphorylation of Raf peptides by the PKA and Akt kinases at serine residues 259 and 365, respectively. This is known to cause inhibition of Raf as was observed in the present experiments (Figure 3). In turn, this is in the agreement with the observed downregulation of MEK activity. Despite the reduced activity of MEK we still noted activity of ERK (Figure 3), which implies that ERK can be activated independently of Raf and MEK upon phagocytosis of *S. aureus*. A Ras-Raf-MEK-independent activation of ERK was proposed in studies on the cellular responses to *Chlamydia trachomatis* infection. In these studies it was shown that increased phosphorylation (and inhibition) of Raf at serine-259 caused increased pathogen growth inside HeLa cells [144]. Non-linear activation of ERK independently of the Ras-Raf-MEK pathway also seems to occur during infection with *Mycobacterium leprae* [146]. Together, these findings support the view that *S. aureus* is able to manipulate pro-apoptotic defence mechanisms by activating the pro-survival ERK cascade to its own benefit [147].

Another important group of kinases, which were activated upon exposure of THP-1 cells to *S. aureus*, are the cytoskeletal kinases of the Src family of tyrosine kinases (Src, Fyn, Lyn and Lck) and Syk (Figures 3 and 4). We observed that the activity of Fyn, Lyn and Lck was decreased, whereas the Src and Syk kinases appeared more active when *S. aureus* was present. The Src family kinases and Syk have been reported to play crucial roles in signal transduction through classical immunoreceptors, such as Fc receptors [148]. Interestingly kinase

profiling did not show any phosphorylation or activation of important proteins involved in cytoskeleton rearrangement and phagosome formation, such as paxillin and the focal adhesion kinase.

The kinase profiling results suggest that the PKA, PI3K and PLC could be regulated by G protein-coupled receptors (GPCR) during incubation of THP-1 macrophages with *S. aureus* (Figure 4). However, we also observed reduced phosphorylation of two GPCR receptors, namely the C-C chemokine receptor type 5 (CCR5) and the complement component 5a receptor 1 (C5aR). The CCR5 is known to play a role in *Yersinia pestis* infection as it has been shown that uptake of *Y. pestis* by macrophages lacking this receptor was significantly decreased [149]. The second receptor C5aR plays a significant role in early non-specific immune responses, which include the migration of neutrophils and macrophages to the site of infection, phagocytosis, generation of superoxide anions, and degranulation [150]. C5aR is activated by anaphylatoxin C5a and bacterial excretion products, such as formylated peptides (fMLP). Both chemoattractants bind to C5aR causing its phosphorylation and subsequent activation. The lower phosphorylation of C5aR as observed by kinase profiling might be caused by its binding to the staphylococcal chemotaxis inhibitory protein (CHIPS), which is known to block this receptor thereby inhibiting C5a- and fMLP-induced Ca^{2+} mobilization in neutrophils and monocytes. A function of CCR5 in *S. aureus* recognition has not been described so far. However, it was recently reported that staphylococcal enterotoxin A (SEA), which is encoded by the same SaPI5 pathogenic island as CHIPS in *S. aureus* NCTC 8325, does inhibit CCR5, CCR2 and possibly CCR1 [151]. If so, this suggests that the *S. aureus* cells used in this study did not produce sufficient amounts of CHIPS to inhibit CCR5, even though they did contain SaPI5.

It has been shown that the scavenger receptor CD36 and integrins are also involved in *S. aureus* recognition and uptake (Figure 4). Integrins form a major family of cell-surface-adhesion receptors that bind to extracellular matrix (ECM) proteins, such as laminin, vitronectin, collagen, fibronectin (FN), elastin and fibrinogen [152,153]. Activation of integrins might be caused by ECM-mediated binding to *S. aureus.* Numerous studies have shown that *S. aureus* produces a variety of surface-exposed proteins that can bind to ECMs and these proteins are thought to play a role in host tissue colonization, invasion of host cells, and ingestion by the host [154]. For example, the fibronectin-binding protein (FnBP) is

one of the staphylococcal ECM-binding proteins which, by binding to the host cell α5β1 integrin, forms a bridge between *S. aureus* and the host cell. This will then lead to bacterial uptake by macrophages and activation of integrin signalling. It has been shown that macrophages lacking integrin β1 exhibit defective phagocytosis of *S. aureus* [155,156].

The scavenger receptor CD36 can also mediate *S. aureus* phagocytosis and cytokine production in response to the staphylococcal cell wall component lipoteichoic acid (LTA). CD36 was shown to be required for *S. aureus* internalization and it plays a role as a TLR-independent signalling receptor (Figure 4). Activation of this receptor leads to increased activity of Src kinases (Fyn, Yes, Lyn) and induces proinflammatory downstream signalling via the JNK signalling pathway, which is independent to TLR2/4 and is associated with pathogen phagocytosis [157,158]. Interestingly we did not find any activated kinases involved in Toll-like receptor 2 (TLR2) signalling, which might suggest that TLR2 is not a primary receptor for *S. aureus* recognition under our experimental conditions. After 15 min of incubation, we also observed an increased activity of the ataxia telangiectasia mutated-(ATM) and ATM-Rad3-related (ATR) kinases (Figures 3), which are involved in the DNA damage response (Figure 4). After 1 hour this activity was decreased, but after 24 hours we observe that the amounts of up- and downregulated peptides, phosphorylated by ATM/ATR kinases were equal, suggesting that other kinases counteracting ATM/ATR were activated. Both ATM and ATR are serine-threonine protein kinases that are recruited and activated by DNA double-strand breaks. They phosphorylate several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis. Several of these targets, including p53, CHK2 and H2AX are tumor suppressors. While double-strand breaks (DSBs) have been regarded as a major signal for ATM activation, there is evidence that ATM is also involved in oxidative stress, when ATM/ATR targets are phosphorylated in response to hypoxia and reoxygenation, the condition during oxidative burst [159,160]. Nitric oxide can also induce the activation and phosphorylation of p53 through ATM/ATR during inflammation [161,162].

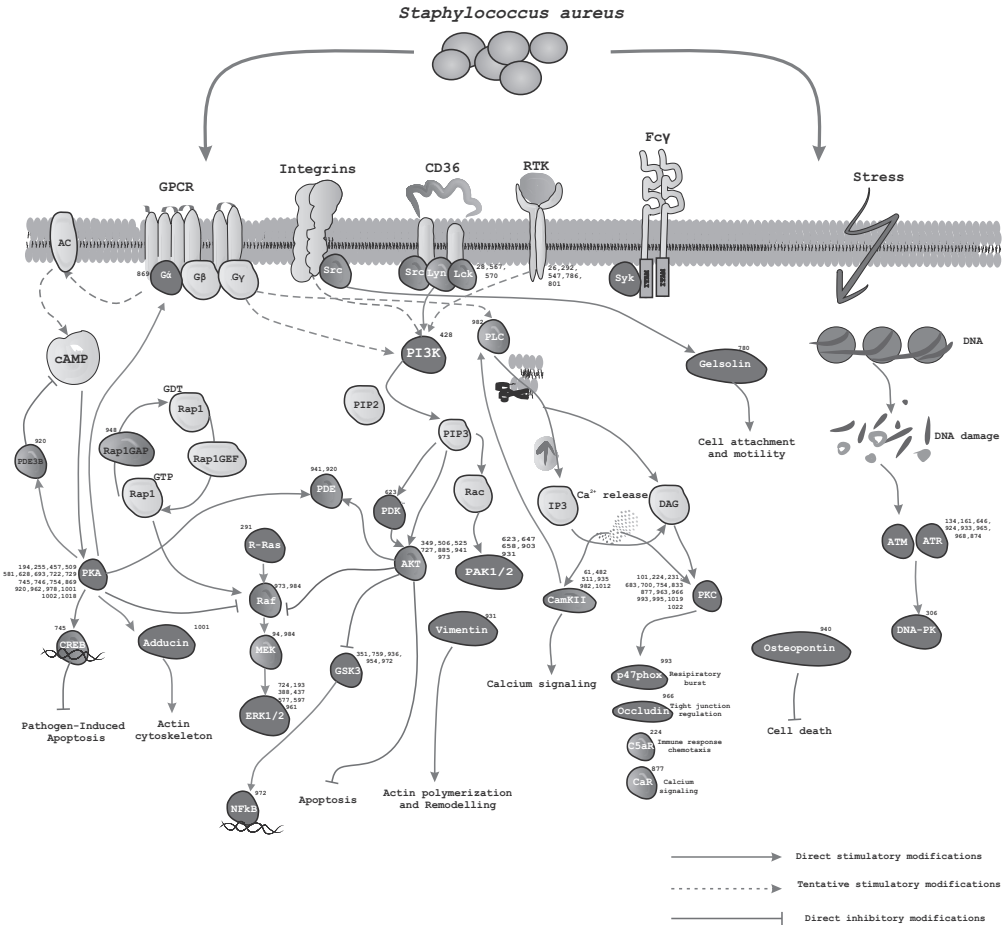

Figure 4. Overview of the signalling pathways active after 15 min incubation of THP-1 cells with *S. aureus*. The kinase profiling results indicate that during incubation with *S. aureus* three main pathways are active in THP-1 cells, namely the cAMP-dependent pathway, the Ca^{2+}-dependent pathway, and the PI3K pathway. These pathways might be regulated during *S. aureus* infection by different receptors such as the G-protein coupled receptor (GPCR), integrins, the scavenger receptor CD36, the receptor tyrosine kinase (RTK), and the Fcγ receptor. For abbreviations of kinases see Results and Discussion. The numbers indicate the Pepscan numbers of peptides in the arrays that were differentially phosphorylated by extracts of THP-1 cells incubated with or without *S. aureus* (for details see Supplementary Table 1 available on JPR website). Kinases that are downregulated are indicated in green, kinases that are upregulated are indicated in red, and kinases that are possibly involved in the active signalling pathways are indicated in yellow. Direct stimulations are indicated by blue arrows, tentative stimulatory effects are indicated by dashed blue arrows, and inhibitory activities are indicated by red inhibition lines.

JNK signalling pathway

Next, we analyzed the signalling cascades in macrophages incubated with *S. aureus* for 1 h. The kinase profiling results show a strong activation of kinases involved in the pro-inflammatory c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways (Figures 3 and 5), and these findings were fully supported by Western Blotting (Figure 6). Activation of the JNK cascade in *S. aureus*-infected macrophages was previously shown to reduce the superoxide level in macrophages and prolonged survival of *S. aureus* in phagosomes. It has also been reported that induction of the JNK pathway is regulated by TLR2 [163]. However, in our experimental setup we did not observe an activation of the TLR2 signalling cascade. Instead, we observed a two-fold increase in the phosphorylation of p90RSK (Figure 3), a transcription factor activated in the Ras-Raf-MEK-ERK pathway in response to reactive oxygen species (ROS). It has been reported that the Fyn kinase is also required for activation of p90RSK [164], which is in agreement with our results that show a significantly increased activity of Fyn (Figure 3). Interestingly, activation of p90RSK was shown to lead to increased survival of *Candida albicans* in mouse macrophages [165] and, judged by our findings, the same might be true for *S. aureus*. Consistent with this view, we observed significant survival rates of *S. aureus* inside THP-1 macrophages even after 24 h incubation (Figure 2C).

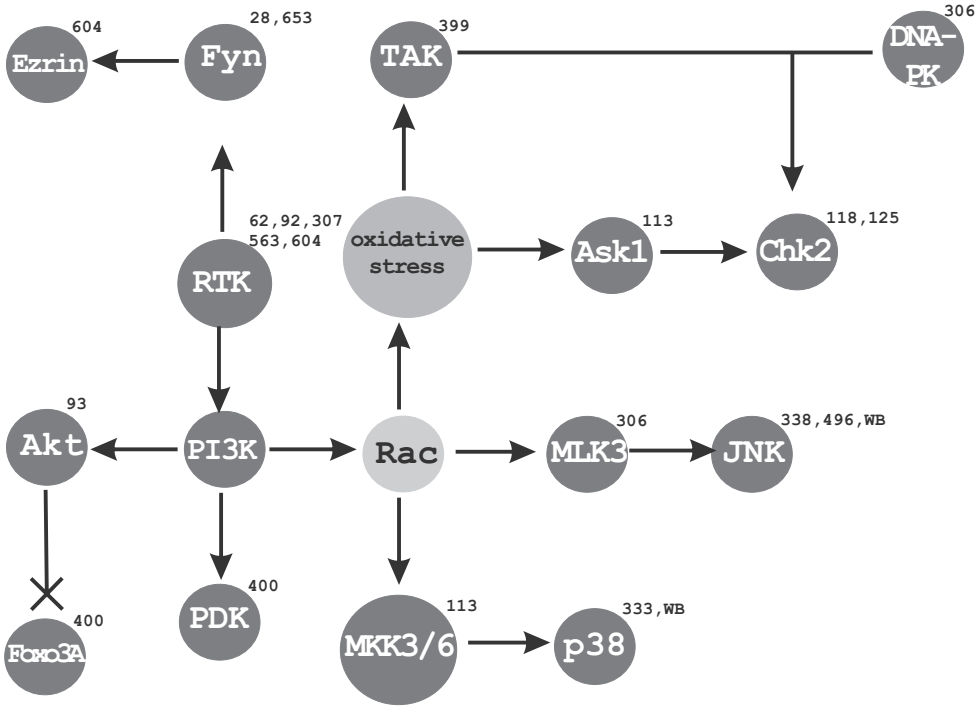

Figure 5. Activation of the JNK and p38 MAPK pathways after 1 hour incubation of THP-1 cells with *S. aureus*. The scheme represents kinases that are potentially activated by oxidative stress during *S. aureus* phagocytosis. The numbers next to the kinase names indicate the Pepscan numbers of peptides in the arrays that were differentially phosphorylated by extracts of THP-1 cells incubated with or without *S. aureus* (for details see Supplementary Table 2 available on JPR website).

Late response of macrophages towards *S. aureus*

To study signal transduction in macrophages incubated with *S. aureus* for 24 h, we incubated THP-1 macrophages with *S. aureus* for 1 h. Next, we removed the extracellular bacteria, washed the macrophages, added gentamicin and incubated the macrophages with internalized bacteria for another 23 h. Then we performed a kinase profiling analysis on the macrophages (summarized in Figure 1). After 24 hours incubation we observed decreased activity of the cAMP-dependent PKA and Akt kinases, which are known to inhibit apoptosis (Figure 3). Interestingly, we also noted increased phosphorylation of peptides phosphorylated by tyrosine kinases (Syk, Lck and Src), receptor tyrosine kinases (PDGFR and INSR), and other kinases involved in cytoskeletal rearrangement and phagocytosis. We observed 2.5-fold and 2.1-fold increase in phosphorylation of the breast tumor kinase (Brk) and the epidermal growth factor receptor (EGFR) peptides. Brk is the tyrosine kinase upregulated in metastatic breast tumors [166]. Recently, Brk has been reported to be activated by EGFR and to promote cell migration and invasion [167]. Moreover, we observed increased phosphorylation of the C1R and C3 complement peptides (2.0 and 1.8-fold) and the tumor suppressing oxidoreductase WWOX (1.7-fold). It has been previously reported that components of the complement system can induce apoptosis, and that this requires activation of the WWOX by C1R [168]. Additionally, Hanayama *et al.* have recently shown that the complement system is also involved in local opsonisation for removal of apoptotic cells by macrophages at sites of inflammation [169]. Taken together, the observed changes in anti-apoptotic signalling pathways and pathways regulating cytoskeletal activity suggest that after 24 hours of incubation some of the infected macrophages start to go into apoptosis. Such apoptotic macrophages might then be phagocytosed by non-apoptotic macrophages. However, no clear decrease in the numbers of macrophages was observed within 24 h incubation in the presence of *S. aureus* (Supplementary Figure 2 - please see JPR website).

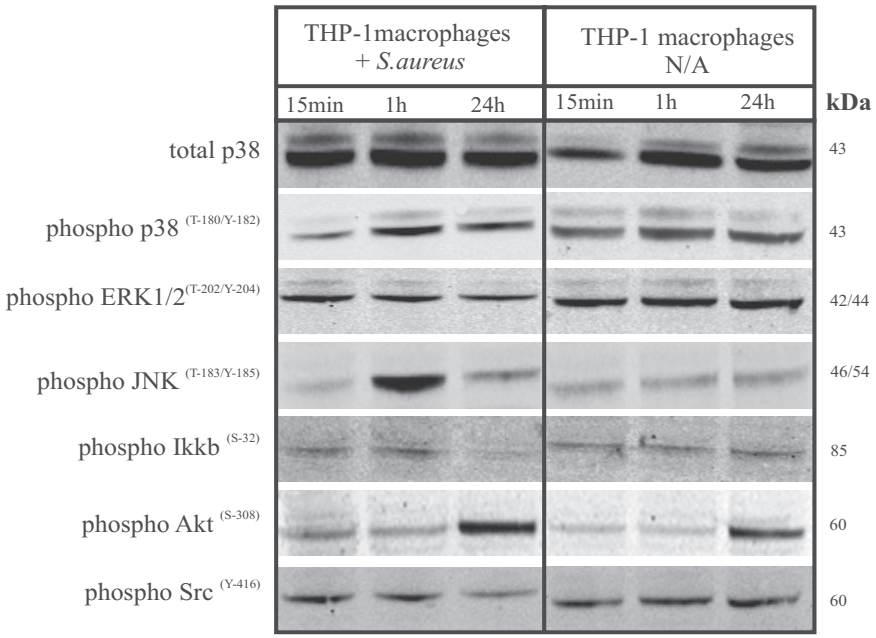

Figure 6. Western blotting controls for *S. aureus*-induced changes in peptide phosphorylation. THP-1 macrophages were incubated for 15 min, 1 h or 24 h with *S. aureus* NCTC 8325. As a control THP-1 macrophages were incubated in the absence of *S. aureus* (N/A). All THP-1 lysates used for Western blotting relate to the same samples that were used for kinase profiling. The membranes were probed with antibodies that are specific for the phosphorylation state of the respective proteins. The apparent molecular weight of each protein is indicated.

Identification of *S. aureus* proteins

To study the responses of *S. aureus* to the THP-1 human macrophages, we performed a gel-free proteomic analysis on non-phagocytosed *S. aureus* cells incubated for 1 h with and without THP-1 cells. The detailed data of the proteomics analyses are provided in Supplementary Table 4, and these results are summarized in Supplementary Table 5 and Figure 7. After 1 h incubation of *S. aureus* with THP-1 cells, we noticed a strong decrease of proteins involved in translation and a concomitant increase of proteins responsible for cell metabolism (Figure 7B, Supplementary Table 5). Thus, the bacteria incubated with macrophages seem to show a "stringent response" due to nutritional stress. This stringent control is one of the most intensively studied systems of gene regulation in bacteria. It is provoked by a rapid accumulation of the alarmones GDP 3'-diphosphate or GTP 3'-diphosphate ((p)ppGpp) upon nutrient limitation. The proteins responsible for the synthesis of ((p)ppGpp) are RelA and SpoT. Alarmone accumulation causes rRNA and tRNA degradation, and over-expression of genes

involved in amino acid biosynthesis to help bacteria survive under stress conditions. Moreover, it has been recently reported that RelA is the key enzyme of the stringent response in *Bacillus subtilis*, which plays a prominent role in nutritional stress activation of σ^B [170]. Consistent with the *B. subtilis* data, our results also reveal the activation of σ^B in *S. aureus* cells incubated with macrophages as exemplified by the detection of elevated levels of the RsbV and RsbW proteins and subunits of the ATP-dependent Clp protease (Supplementary Table 5) [171]. Moreover, we also observed the increase of proteins involved in the response to oxidative stress (*e.g*. thioredoxin and the redox-sensing transcriptional repressor Rex), protein secretion (*e.g*. the foldase PrsA and the preprotein translocase subunit SecY), and heme biosynthesis (*e.g*. ferrochelatase and the delta-aminolevulinic acid dehydratase) (Supplementary Table 5 available on JPR website).

Notably, in the samples of *S. aureus* incubated with macrophages, we detected two phenol-soluble modulins (PSMs, Q2FZA4 and Q2FZA3, Supplementary Table 5). PSMs are important virulence factors of *S. aureus* that lyse neutrophils and other cell types, and that elicit a series of proinflammatory responses. Furthermore, it has been reported that PSMs from *Staphylococcus epidermidis* induce production of the tumor necrosis factor-alpha (TNF-alpha) and the transcription factor nuclear factor κB (NF-κB) in THP-1 macrophages [172,173]. Binding of TNF-alpha to TNF receptors (TNF-R1 and/or TNF-R2) can lead to activation of the JNK and p38 pathway. Therefore, activation of JNK and p38 MAP kinases, as it was shown in our kinase profiling data might be influenced by the presence of PSMs and subsequent production of TNF-alpha.

S. aureus cells grown in the presence of THP-1 cells were shown to produce the staphylococcal serine/threonine kinase PknB (Q2FZ64; also known as Stk1, Supplementary Table 5), whereas this protein was not detectable in cells incubated without THP-1 cells. PknB is a staphylococcal serine/threonine kinase involved in cellular and cell wall metabolism, antibiotic resistance and virulence [103,174]. We have recently shown that PknB can be released from the bacterial cells into the extracellular milieu. This finding opens up the possibility that human proteins are phosphorylated by PknB, for example when *S. aureus* is ingested by host cells [117]. Consistent with this view, PknB was recently detected in cells of *S. aureus* RN1HG that were internalized by human airway epithelial cells [175].

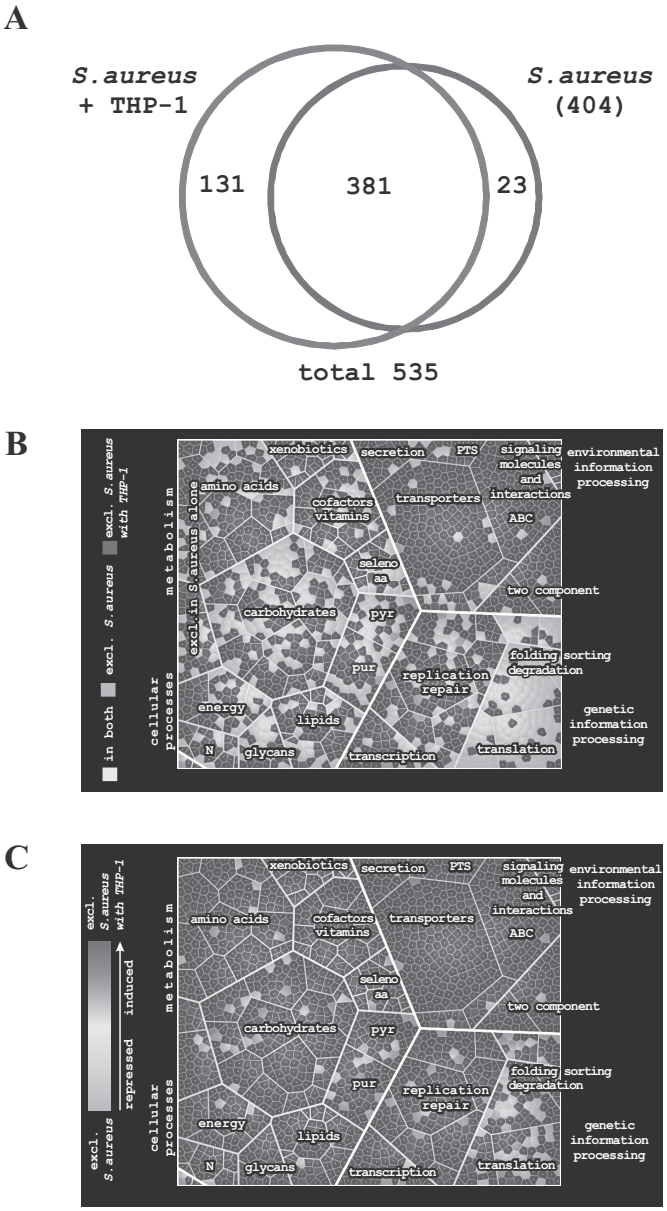

Figure 7. Overview of identified *S. aureus* proteins. (A) The Venn diagram shows the numbers of identified peptides of proteins from *S. aureus* NCTC 8325 that were detected when *S. aureus* was cultivated in the presence or absence of THP-1 macrophages. The Venn diagram was generated using the BioInfoRX Venn diagram plotter (http://bioinforx.com/free/bxarrays/venndiagram.php#). (B) Voronoi treemap based on the KEGG gene orthology for *S. aureus*, visualising the function of the identified proteins. Each small field represents an independent protein. Proteins identified exclusively in *S. aureus* cells cultured in RPMI are indicated in green, proteins found exclusively in *S. aureus* cultured in RPMI in presence of THP-1 macrophages are indicated in red, and proteins present in both conditions are indicated in yellow. Proteins labelled in grey were not detected. (C) Changes in the amounts of *S. aureus* proteins upon incubation with THP-1 cells. Reduced protein levels are indicated in shades of green, and increased levels are indicated in shades of red.

Binding of human proteins to *S. aureus*

To identify human proteins that bind to *S. aureus*, we incubated THP-1 macrophages with *S. aureus*. After 1 h, we collected non-phagocytosed *S. aureus*, disrupted the cells and performed MS analysis. The obtained MS data was searched against a database with human proteins. The results showed that during incubation with the THP-1 macrophages several human proteins were bound by *S. aureus* (Table 1 and Supplementary Table 6 available on JPR website).

Two proteins that were bound by *S. aureus* are so-called heat shock proteins (HSPs) of the HSP60 and HSP70 type. Recent studies showed that HSPs are not only induced by heat, but also by other stimuli such as growth factors, inflammation, and infection [176]. Human HSP60 and HSP70 were reported to activate macrophages and dendritic cells via TLR4 and/or TLR2 [177]. Srivastava *et.al* have shown that HSPs are endogenous adjuvants, which can be used to induce strong tumor- or pathogen-specific immunity [178,179]. Moreover GP96 or HSP70 were shown to induce NO production in murine and human macrophages [178]. There are several possible explanations why the HSPs were found in association with *S. aureus* cells. For example, some of the *S. aureus* cells may have lysed during the incubation with THP-1 cells leading to the release of DNA into the extracellular milieu. If so, unmethylated CpG motifs in bacterial DNA may have triggered the release of HSP70 and an inflammatory response [180]. This would be consistent with the observed upregulation of Rac, JNK, and p47phox (Figures 3, 4 and 5). Furthermore, binding of HSP60 to both staphylococcal FnBP and human fibronectin was shown to mediate staphylococcal internalization by epithelial cells [181]. More recently, the heat shock cognate protein Hsc70 was shown to bind the major *S. aureus* autolysin Atl thereby functioning as a host cell receptor in epithelial cells [175]. Indeed, we identified Atl in all staphylococcal samples so, by analogy, this protein might be involved in HSP70 binding.

Table 1. Human proteins bound to *S. aureus* NCTC 8325 incubated with THP-1 macrophages.

Accession	Identified Proteins
IPI00026271	40S ribosomal protein S14
IPI00221092	40S ribosomal protein S16
IPI00021439	Actin, cytoplasmic 1
IPI00008274	Adenylyl cyclase-associated protein 1
IPI00020599	Calreticulin precursor
IPI00299571	CDNA FLJ45525 fis, clone BRTHA2026311, highly similar to Protein disulfide isomerase A6
IPI00015911	Dihydrolipoyl dehydrogenase, mitochondrial precursor
IPI00465248	enolase 1
IPI00465439	Fructose-bisphosphate aldolase A
IPI00472102	Heat shock protein 60
IPI00410714	Hemoglobin subunit alpha
IPI00479217	Heterogeneous nuclear ribonucleoprotein U isoform b
IPI00217465	Histone H1.2
IPI00018534	Histone H2B type 1-L
IPI00453473	Histone H4
IPI00009865	Keratin, type I cytoskeletal 10
IPI00217963	Keratin, type I cytoskeletal 16
IPI00019359	Keratin, type I cytoskeletal 9
IPI00220327	Keratin, type II cytoskeletal 1
IPI00021304	Keratin, type II cytoskeletal 2 epidermal
IPI00009867	Keratin, type II cytoskeletal 5
IPI00335168	myosin, light chain 6, alkali, smooth muscle and non-muscle isoform 1
IPI00026519	Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor
IPI00216691	Profilin-1
IPI00010796	Protein disulfide-isomerase precursor
IPI00784179	pyruvate kinase 3 isoform 2
IPI00007765	Stress-70 protein, mitochondrial precursor
IPI00216298	Thioredoxin
IPI00024919	Thioredoxin-dependent peroxide reductase, mitochondrial precursor
IPI00550363	Transgelin-2
IPI00166768	TUBA6 protein

A second class of human proteins that we found to bind to *S. aureus* cells were histones. Histones are alkaline nuclear proteins that package and order the eukaryotic DNA into structural units called nucleosomes. Interestingly, the histones H1.2 and H4 were shown to have antimicrobial activity against *S. aureus* and several other bacteria [168,182], and the histone H1 was shown to protect mice against infection with *Leishmania major* [183,184]. Moreover, histones and antimicrobial peptides are also components of so called extracellular traps (ETs). ETs are formed by phagocytes after stimulation with mitogens, cytokines or pathogens themselves, and upon induction of ROS. ETs consist of nuclear or mitochondrial DNA as a backbone with antimicrobial peptides, histones, and cell-specific proteases. It has been shown that ETs are formed to entrap and kill microbes, such as *S. aureus* [185,186], *Streptococcus pyogenes* [187], and *Streptococcus pneumoniae* [188]. Notably, while the inclusion of histones in ETs can have beneficial effects in combating infection, histones can also be very detrimental. For example, they were shown to be toxic for endothelial cells *in vitro*, and injection of histones into mice caused the symptoms of sepsis, including organ failure [189]. Finally, histone-like proteins have recently been implicated in the adherence of *Streptococcus gallolyticus* to colon adenocarcinoma cells [190]. While we did not detect such proteins from *S. aureus*, the identification of human histones bound by *S. aureus* opens up the possibility that these histones serve a role in staphylococcal adhesion to host cells.

Since there was a possibility that certain FCS proteins present in the growth medium might also bind to *S. aureus*, we compared the results from searches against human and bovine databases. Indeed, we identified five peptides representing four different bovine proteins in the samples of *S. aureus* cells grown in medium supplemented with 10% FCS (no THP-1 macrophages present). In contrast, the samples from the bacteria that were challenged with macrophages and grown in RPMI supplemented with 10% FCS resulted in the identification of 45 potentially 'bovine' peptides corresponding to 36 proteins (Supplementary Table 6). For 24/22 of these peptide/proteins, the peptide identifications in the bovine database can be explained by their homology with human proteins. Moreover, for three of these proteins we also identified peptides that were specific for the respective human proteins. We therefore conclude that at least these three proteins originate from the macrophages and not from the FCS. Furthermore the search against the bovine database resulted in 21/14 truly

bovine-specific peptide/proteins identifications. A possible explanation why these bovine proteins were only identified in samples of *S. aureus* cells incubated in the presence of THP-1 macrophages is that the macrophage challenge may have induced changes in the staphylococcal cell surface that then promoted the binding of FCS proteins. Alternatively, the bovine proteins associated with human proteins that were bound to the staphylococcal cell surface.

Conclusion

Taken together, our present studies represent a first snapshot of the complex phosphorylation networks that are triggered in human macrophages encountering staphylococcal cells. Conversely, the proteomics data provide novel insights into the responses of *S. aureus* to the presence of macrophages, and possible interactions between staphylococcal cells and human proteins. The results reveal an intricate network of signalling events mediating responses to *S. aureus*, markedly different from the classical view in which a canonical pathway acting through TLR receptors and NF-kB coordinates the host response. The integrated approach pursued in the current study thus challenges the classical concept that such macrophage responses are mainly mediated through TLR and NF-kB signalling and highlight the complexity of signalling cassettes orchestrating host defence.

Materials and Methods

Bacterial strains and growth conditions

For invasion experiments, *S. aureus* strain NCTC 8325 was grown overnight at 37°C in 10 ml of Trypticase Soy Broth (TSB). The overnight cultures were then diluted to an initial optical density at 600 nm (OD_{600}) of 0.08 in 15 ml of TSB and grown at 37°C to an OD_{600} of 0.8, which corresponds to 2.5×10^8 colony forming units (CFU). Bacteria were washed three times in phosphate-buffered saline (PBS) and resuspended in the invasion medium RPMI (Gibco) supplemented with 2 mM L-glutamine (PAA) and 10% heat-inactivated Fetal Bovine Serum (FBS; PAA).

Cell culture and growth conditions

THP-1 cells [191] were grown in suspension in RPMI 1640 medium (Gibco) supplemented with 2 mM glutamine (PAA) and 10% heat-inactivated FBS (PAA). Cell culturing and all subsequent experiments were carried out at 37°C under 5%

CO_2. Cells were passaged every three days. Cell viability as determined by the trypan blue exclusion assay [192] was at least 90% before and during all experiments.

For kinase profiling assays, 10^5 cells were seeded in 6-well plates, and 10^7 cells were seeded in 150 cm^2 flasks (TPP) for proteomics experiments. Differentiation and adherence of THP-1 monocytes was induced by incubation with 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) for 2 days at 37°C in 5% CO_2 [193]. After 2 days of differentiation, the medium was removed, cells were washed three times in PBS, and then the cells were incubated for one more day in fresh supplemented RPMI.

Infection experiments for kinase profiling

THP-1 macrophages were infected with *S. aureus* NCTC 8325 at a multiplicity of infection (MOI) of 1:50. Macrophages were incubated with bacteria for 15 min, 1 h or 24 h (schematically represented in Figure 1). As a negative control macrophages without bacteria were used and in all subsequent steps these macrophages were treated in the same way as the infected macrophages. After 1 h, medium with non-internalized bacteria was removed, cells were washed three times in PBS, and fresh RPMI 1640 medium supplemented with 2 mM glutamine, 10% heat inactivated FCS and 100 µg/ml gentamicin was added and macrophages were incubated for another 23 h. At each time point of sampling (15 min, 1 h, 24 h), the medium was removed and THP-1 cells were washed three times in PBS (PAA). The cells were then lysed with Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific), and incubated with Halt Protease and Phosphatase Inhibitor Cocktails (Thermo Scientific) for 1 h on ice under gentle shaking. Lysed cells were scraped from the plates, collected in fresh Eppendorf tubes and centrifuged for 10 min (14000 rpm, 4°C) in order to remove cellular debris. Then the supernatant was collected and the protein concentration was determined using the Micro BCATM Protein Assay Kit (Thermo Scientific). Furthermore, at each time point, aliquots of the macrophage lysates and the macrophage growth medium were plated to determine the colony forming units of internalized and extracellular *S. aureus*.

Scanning electron microscopy

For scanning electron microscopy, *S. aureus* was incubated with THP-1 macrophages for 15 minutes. Samples were fixed for 30 min with 2% glutaraldehyde in 0.1 M Cacodylate buffer, pH 7.38. The fixed samples were placed on a piece (1 cm^2) of cleaved 0.1% Poly-L Lysine coated mica sheet and washed in 0.1 M Cacodylate buffer. The specimen were then dehydrated in ethanol series consisting of 30%, 50%, 70%, 96% and anhydrous 100% solutions (3×) 10 min each, then critical point dried with CO_2, and sputter-coated with 2-3 nm Au/Pd (Balzers coater). The specimen were fixed on a SEM-stub-holder and observed in a JEOL FE-SEM 6301F microscope.

Kinase profiling array analyses

The specificity of kinases to their substrates is dependent on a multitude of factors, like their physical localisation, but a very important factor is the amino acid context surrounding the substrate's threonine, serine, or tyrosine residues that are to be phosphorylated. This characteristic of kinases is exploited in kinase profiling with peptide arrays. In our approach, we employed the entire complement of kinase substrates described in Phosphobase [194], which were spotted on hydrogel-coated glass as described by Diks *et al* [116]. This resulted in slides that display 1,152 individual kinase substrates (*i.e.* peptides) in duplicate. The sequences of these peptides and the source proteins from which they were derived can be found in the Supplementary Tables 1-3 available on JPR website and, in more detail, on http://www.pepscanpresto.com/files/PepChip%20Kinase%20Map%20File%200103.xls. Procedures for performing kinase profiling were previously described in detail [116]. In short, cells were lysed in 50 µl lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM $MgCl_2$, 1 mM glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/ml Leupeptin, 1 µg/ml Aprotinin, 1 mM PMSF). Immediately before the actual profiling, 10 µl of the peptide array incubation mix (50% glycerol, 50 µM ATP, 0.05% v/v Brij-35, 0.25 mg/ml BSA, [^{33}P]-γ-ATP (1 MBq)) was added to the filtered lysates and the mixtures were loaded onto the peptide microarray chips, which were then incubated for 90 min at 37°C. The chips were washed twice with TBS 0.1% Triton X-100, twice in 2 M NaCl, and twice in de-mineralized water before air-drying. The slides were then exposed to a phosphor - imager plate for 72 h and image acquisition of the peptide array was performed

using a phosphor-imager (Storm™, Amersham Biosciences, Sweden). Levels of incorporated radioactivity, which correspond to the phosphorylation status were quantified by the array software Scanalyze (Eisen Software) and exported to Microsoft Excel. Kinase activities in lysates from three time points were each analysed in triplicate. As a control, slides were incubated only with ^{33}P-γ-ATP (1 MBq)), which demonstrated that the observed signals represented covalent transfer of the terminal phosphor atom to peptides on the chip. Examples of kinase slides can be found in Supplementary Figure 1.

For data analysis, first every peptide was given an “on” call or “off” call (Markov state analysis). To this end, first an average signal was calculated for each peptide using the three biological replicates (each consisting of two technical replicates) yielding an aggregate dataset for each individual experiment. Subsequently, for each of the aggregate datasets, either “on” calls or “off” calls were given to each peptide substrate (Markov state analysis). In order to do this, we assumed that the subset of signals representing the $1\text{-}e^{-1}$ fraction of peptides having the lowest phosphorylation of all peptides contained pure noise and did represent meaningful phosphorylation. The distribution of this noise was fitted as a single exponent, using the amplitude-sorted row number of these substrates as the X domain of the distribution and this single exponent was assumed to describe noise for the entire dataset in this particular experiment. Now for all data points within the subset, when the actual amplitude observed minus 1.96 the standard deviation was in excess of the value expected from distribution describing the noise, a substrate was given an “on” call. Subsequently, phosphorylation profiles of the different conditions were contrasted. For this analysis values between different sets were compared, and when one set displayed an “on” call, whereas the other set received an “off” call and when the absolute difference in signal was at least 10% of the strongest signal, a peptide was considered to represent a differential kinase activity. The result was then used to construct provisional signal transduction schemes representing the difference in signalling between the two datasets, analogous to the construction of provisional signal transduction schemes described earlier by van Baal *et al*. [195]. Data which met all the above-listed requirements and with p values lower than 0.05 were considered as significant.

Western Blotting

Western Blotting was done as described by Miller *et al*. [196]. Blots were incubated overnight at 4°C with the primary antibody. Antibodies recognizing phosphorylated p38 MAPK$^{Thr180/Tyr1982}$, phosphorylated ERK1/ERK2$^{Thr202/Tyr204}$, phosphorylated JNK$^{Thr183/Tyr185}$, phosphorylated IκB alpha^{Ser32}, phosphorylated Akt^{Thr473} and Akt^{Thr307} were purchased from Cell Signaling (Beverly, CA). Macrophage lysates were mixed with SDS sample buffer and heated at 95°C for 5 min. The subsequent SDS-PAGE and protein transfer to nitrocellulose membranes (Protran®, Schleicher and Schuell) by semi-dry blotting were performed as previously described [117]. Membranes were blocked for 1 h with 5% bovine serum albumin (BSA) in PBS supplemented with 0.1% Tween 20 (PBS-T) and incubated overnight at 4°C in 5% BSA PBS-T with the indicated primary antibodies. Next, membranes were washed three times for 5 min in PBS-T before adding a fluorescent secondary antibody at a 1:20000 dilution in blocking buffer (IRDye 800 CW goat anti-rabbit antibody from LiCor Biosciences). Membranes were incubated for 1 h in the dark, washed three times for 5 min in PBS-T and once in PBS. After transferring the membranes into fresh PBS, they were scanned using the Odyssey Infrared Imaging System (LiCor Biosciences).

Proteomic analysis of *S. aureus* during infection

THP-1 macrophages were incubated with *S. aureus* NCTC 8325 at MOI 1:50 for 1h. As a control we used *S. aureus* in RPMI 1640 (2 mM glutamine and 10% heat inactivated FCS) without macrophages (see schematic representation of the experimental setup in Figure 1). After 1 h, medium with non-internalized bacteria was collected and bacteria were centrifuged for 5 min (3750 rpm, 4°C). The supernatant was removed and bacterial cells were washed three times in PBS and once in PBS with 20 mM sodium azide. Washed bacterial cells were resuspended in M-PER, disrupted using a Precellys 24 bead beater (Bertin Technologies; three runs of 30 s, 6800 rpm) and centrifuged for 5 min (14000 rpm, 4°C). The supernatant was collected and the protein concentration was determined using the Micro BCA™ Protein Assay Kit. 10 µg of lyophilized protein was solubilized with the acid labile detergent RapiGest SF (Waters Corp., Milford, MA). Activated sequence grade trypsin (Promega, Madison, WI) was added and incubated overnight at 37°C. The detergent was removed by acidification of the solution and subsequent centrifugation. The peptides thus obtained were subjected to

reversed phase chromatography performed on a nanoACQUITY UPLC system (Waters Corporation, Milford, Mass., USA) with an on-line coupled mass spectrometric (MS) analysis by an LTQ Orbitrap (Thermo Fisher, Bremen, Germany). Desalting and concentration of peptides was performed on a trap column (Waters nanoACQUITY UPLC column, Symmetry C18, 5 µm, 180 µm x 20 mm, Waters Corporation, Milford, Mass., USA) by loading and washing for 3 min with 99% buffer A (0.1% v/v acetic acid) at a flow rate of 10 ml/min. Peptides were subsequently eluted onto an analytical column (Waters BEH 1.7 µm, 100-µm i. D. x 100 mm, Waters Corporation, Milford, Mass., USA) and separated with a non-linear 80 min gradient from 5-60 % acetonitrile in 0.1 % acetic acid at a constant flow rate of 400 nl/min. MS and MS/MS data were acquired with the LTQ-Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany) equipped with a nanoelectrospray ion source. After a survey scan in the Orbitrap (r= 30,000) MS/MS data were recorded for the five most intensive precursor ions in the linear ion trap. Singly charged ions were not taken into account for MS/MS analysis. Tandem mass spectra were extracted using Sorcerer v3.5 (Sage-N Research). All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version v.27, rev. 11). Database searching was performed against a target decoy database of concatenated staphylococcal proteins (extracted uniprot database for strain NCTC 8325) with added common laboratory contaminant proteins (5866 entries). Cleavage specificity for full tryptic cleavage and a maximum of 2 missed cleavages was assumed. Sequest was run with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10 ppm. Oxidation of methionine was specified in Sequest as a variable modification. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides, which resulted in a false discovery rate (FDR) below 1% for peptide identifications for the bacterial data base searches. An additional search was performed against the human IPI database containing the reverted protein sequences (138660 entries). Identifications of the human data set needed XCorr scores greater than 1.9, 2.8, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides, which resulted in an FDR below 1% for peptide identifications. In addition a search was performed against the bovine database containing also the reverted protein sequences (47308 entries). Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9,

3.0, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides. The filter criteria were adjusted in the way that the FDR was <1%. A protein identification was accepted if the respective protein was detected in at least three out of four biological replicates.

Proteins that were identified in at least three of the four replicates of one condition, but in none of the replicates of the second conditions were regarded as “on” and “off” calls, respectively. For the proteins that were identified in both conditions the natural logarithm was calculated for the un-weighted spectral counts and the ratios of the bacterial samples that were challenged with THP-1 cells and the bacterial control samples were calculated. The ratios were then normalized to the median and the average as well as the standard deviation was calculated for every protein. A protein was regarded as being present in different amounts in the THP-1-challenged bacterial samples in comparison to the control samples when the average ratio exceeded the standard deviation at least two-fold and the fold change was determined using the normalized normal values.

Chapter 3

Staphylococcal PknB as the first prokaryotic representative of the proline-directed kinases

PLoS ONE 5(2): e9057. doi:10.1371/journal.pone.0009057

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Abstract

In eukaryotic cell types, virtually all cellular processes are under control of proline-directed kinases and especially MAP kinases. Serine/threonine kinases in general were originally considered as a eukaryote-specific enzyme family. However, recent studies have revealed that orthologues of eukaryotic serine/threonine kinases exist in bacteria. Moreover, various pathogenic species, such as *Yersinia* and *Mycobacterium*, require serine/threonine kinases for successful invasion of human host cells. The substrates targeted by bacterial serine/threonine kinases have remained largely unknown. Here we report that the serine/threonine kinase PknB from the important pathogen *Staphylococcus aureus* is released into the external milieu, which opens up the possibility that PknB does not only phosphorylate bacterial proteins but also proteins of the human host. To identify possible human targets of purified PknB, we studied *in vitro* phosphorylation of peptide microarrays and detected 68 possible human targets for phosphorylation. These results show that PknB is a proline-directed kinase with MAP kinase-like enzymatic activity. As the potential cellular targets for PknB are involved in apoptosis, immune responses, transport, and metabolism, PknB secretion may help the bacterium to evade intracellular killing and facilitate its growth. In apparent agreement with this notion, phosphorylation of the host-cell response coordinating transcription factor ATF-2 by PknB was confirmed by mass spectrometry. Taken together, our results identify PknB as the first prokaryotic representative of the proline-directed kinase/MAP kinase family of enzymes.

Introduction

Despite their clinical relevance, the mechanisms employed by pathogenic bacteria to subvert the host immune system remain only partially characterised. It has become clear, however, that pathogens create a beneficial environment for their survival by secreting proteins that mimic the functions of several host proteins. One of the best known bacterial examples is *Yersinia pestis*, the plague bacterium, which injects its effector proteins into the host cells by the type III secretion system [197,198]. These *Yersinia* effector (Yop) proteins include the eukaryotic-like serine/threonine kinase YpkA, also known as YopO [199]. This kinase shows a high degree of sequence similarity to mammalian serine/threonine protein kinase domains. YpkA is translocated into a host cell where it disrupts the actin-based cytoskeletal system and promotes both survival and replication of bacteria by an unknown mechanism [199,200,201]. Nevertheless, the full spectrum of human proteins that are phosphorylated by YpkA has remained elusive so far [202].

Eukaryotic-like serine/threonine protein kinases (STPKs) are present not only in the *Yersinia* genus, but they have also been identified in the soil microorganism *Myxococcus xanthus* [203,204] and in human pathogens, such as *Mycobacterium tuberculosis*, which even encodes 11 STPKs. Only two of these (PknG and PknK) are soluble proteins, while the other nine STPKs contain a transmembrane domain [205]. Moreover STPKs have also been identified in *Pseudomonas aeruginosa* [206,207], *Streptococcus pneumoniae* [208,209,210] and in *Staphylococcus aureus* [104,106,211]. However, the precise biological functions and substrate specificities of these kinases have not yet been defined.

Recently, attention has been focussed on the PknB kinase of *S. aureus.* This Gram-positive bacterium is part of the human microbiota, but it can turn into a dangerous pathogen, causing a wide range of infections [2,3,4]. Although *S. aureus* is mostly considered as an extracellular pathogen, it can invade a variety of mammalian non-professional cells, such as nasal endothelial cells. Moreover *S. aureus* survives phagocytosis by professional phagocytes [212,213,214], such as neutrophils [62,65], mouse or rat macrophages [215,216,217,218], and human macrophages [66,219]. To overcome the stressful conditions imposed by its host, *S. aureus* has evolved various protective and offensive responses [115,154,220,221], such as sensing of environmental stimuli and the activation and inactivation of response regulators [147,222]. This is generally achieved

through cascades of phosphorylation reactions in the host, which focuses a strong interest on the role of kinases, such as the serine/threonine kinase PknB (also known as StpK) in staphylococcal persistence.

The PknB kinase is composed of three extracellular PASTA domains (penicillin binding domains), a central transmembrane domain and an intracellular kinase domain [211]. Interestingly, it was recently reported that PknB is not only involved in regulation of the central metabolism of *S. aureus* [104], but also determines staphylococcal infection of mouse kidneys in an abscess model [106]. The latter observation raises the question whether the kinase activity of PknB is directly or indirectly involved in the pathogenicity of *S. aureus*. A direct role of PknB in infection is conceivable since serine/threonine kinases play key roles in mammalian cell signalling, and at least two bacterial equivalents, YpkA of *Yersinia* and PknG of *Mycobacterium tuberculosis*, have been shown to be directly involved in the subversion of host cells during the respective infectious processes [223,224]. However the exact role played by PknB in pathogenesis or staphylococcal persistence has thus far remained unclear.

Here we show that full-size soluble PknB is present in the medium of growing *S. aureus* cells. We therefore investigated whether PknB of *S. aureus* can recognize and phosphorylate known substrates of human serine/threonine kinases. For this purpose, we used peptide microarrays with known human phosphorylation sites. The phosphorylation profile and mass spectrometry results show that PknB is a proline-directed kinase, which can indeed phosphorylate specific human targets. The observed target specificity of PknB indicates possible roles for this enzyme in a wide range of host cell signalling processes during *S. aureus* infection.

Results and Discussions

Identification of extracellular PknB

It has previously been reported that different bacteria such as *M. tuberculosis* and *Yersinia* species can secrete their eukaryotic-like serine/threonine kinases directly into the host. This mechanism allows these bacteria to survive intracellularly [205,223], to disrupt the actin cytoskeleton [225], or to cause host cell apoptosis [224]. Since these bacterial ser/thr kinases need to be exported in order to impact on host cells, we investigated whether PknB might be detectable in the extracellular milieu of *S. aureus*. As shown by Western blotting using polyclonal antibodies against PknB, the full-size PknB was detectable both in the cellular and growth medium fractions of cells of *S. aureus* NCTC 8325 harvested at an OD_{600} of 2. As expected, PknB was neither detectable in cellular nor growth medium fractions of the Δ*pknB* mutant. The precise mechanism by which PknB is liberated from the wild-type cells remains to be elucidated. However, there is precedence for the release of membrane proteins, or fragments thereof, into the extracellular milieu of Gram-positive bacteria, such as *S. aureus*, by as yet unknown mechanisms [226]. One possibility is that these proteins are released by cell lysis, remaining stable in the medium due to an intrinsic resistance to extracytoplasmic proteolysis [227]. The idea that PknB is released by lysis would be consistent with the detection of relatively small amounts of the cytoplasmic marker protein thioredoxin A (TrxA) in the growth medium fractions (Fig. 1). TrxA is a cytoplasmic bacterial protein, which acts as an antioxidant by facilitating the reduction of cysteine disulfides in other cytoplasmic proteins. Since TrxA is normally a cytoplasmic protein, it will only be found in the extracellular milieu when bacterial cells have lysed. Notably, compared to the cellular samples, we detected relatively more extracellular PknB than extracellular TrxA, which might suggest that the release of PknB is the consequence of a specific process rather than cell lysis. Irrespective of the mechanism by which PknB is released into the extracellular milieu of *S. aureus*, its release may impact on human host cell functions. This could be the case upon internalization of *S. aureus*. Although *S. aureus* is primarily an extracellular pathogen, there is strong evidence that it can be internalized by a wide range of human host cells. For example, *S. aureus* invades non-professional phagocytes by a mechanism which requires a specific interaction between the bacterial fibronectin-binding protein and the host cell [228,229,230]. This leads to

host signal transduction, activation of tyrosine kinases, cytoskeletal rearrangement and endosome uptake. Bayles and Qazi reported that internalized *S. aureus* is able to escape from the host endosome, and this fact opens up the possibility of direct interactions of released PknB with proteins of the human host [231,232].

Eukaryotic phosphorylation sites recognized by PknB

Peptide arrays (PepChips) have previously been used successfully to profile the activity of kinases in eukaryotic cell lysates [116]. We therefore employed this array-based technology to investigate whether the staphylococcal kinase PknB has the ability to recognize and phosphorylate human phos-phorylation sites. When the PepChips were incubated with purified and active PknB [211] and [^{33}P-γ] ATP, radioactivity was efficiently incorporated in a particular subset of the peptides on the chip. In contrast, little radioactivity was incorporated when the arrays were incubated with [^{33}P-γ] ATP in the absence of PknB. We identified 68 potential substrates for PknB, of which the biological functions are summarised in Table 1 (for details, see Table S1 available on http://www.plosone.org/article/info:doi%2F10.1371%2Fjournal.pone.0009057). Interestingly, 32 of the potential human substrates of PknB are involved in signal transduction and cell communication. The identified peptides include serine/threonine kinases, cell cycle control proteins, and regulators of protein phosphorylation such as adaptor molecules. Our results suggest that any active PknB released from invasive *S. aureus* cells may target signal transduction mechanisms for host cell subversion. In addition, 13 potential PknB substrates are involved in gene regulation, including transcription factors, transcription regulatory proteins and RNA binding proteins. Three potential substrates play a role in immune responses and recognition, five in transport processes, ten in cell growth and maintenance (cytoskeletal and structural proteins), two in cell metabolism, two in stress responses, and one in apoptosis.

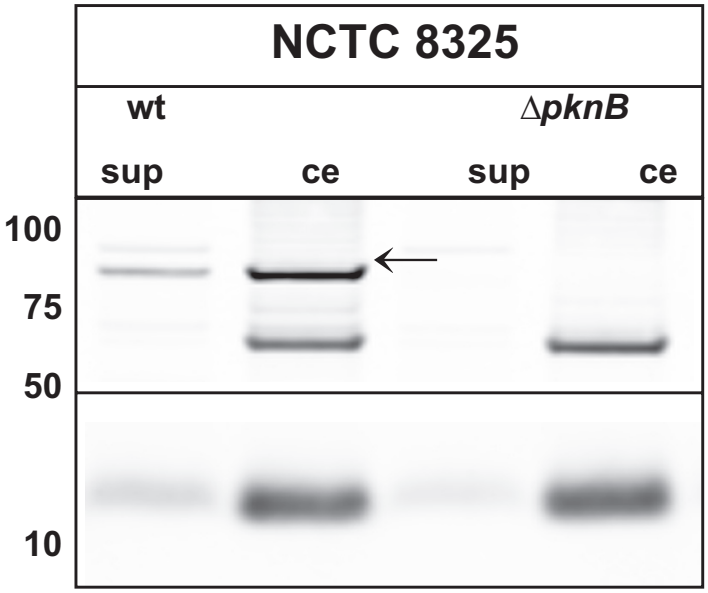

Figure 1. Release of PknB into the growth medium of *S. aureus*. The *S. aureus* strain NCTC 8325 (wt) or a Δ*pknB* derivative were propagated at 37° C in TSB and harvested at OD_{600} 2. Crude extracts (ce) and supernatant (sup) fractions were isolated, corrected for OD and separated by NuPAGE electrophoresis (Invitrogen). Two-fold higher amounts of the supernatant fractions were used for PAGE as compared to the crude extracts. Immunoblotting was conducted using specific antibodies against PknB (upper panel) or TrxA (lower panel). The latter served as an indicator for cell lysis. The position of the specific PknB signal is marked with a black arrow. The band at ~60 kDA corresponds to an unidentified protein, which cross-reacts with the antibodies against PknB. The molecular weight of marker proteins is indicated on the left.

Phosphorylation of the identified potential human PknB targets would result in significant changes in host cell signal transduction. One of the peptides that is best phosphorylated by PknB (^{64}IVADQTPTPTR^{74}) is derived from the Activating Transcription Factor-2 (ATF-2) (see Table S2 available on PLos One website). ATF-2 belongs to the bZIP (Basic Leucine Zipper Domain) DNA-binding protein family and is expressed by almost all human cells (GeneNote, Ensemlb ID: ENSG00000115966). In unstimulated cells, ATF-2 is maintained in an inactive form by interactions between its own activation domain and its bZIP domain [233]. In response to certain stimuli [234,235,236], the kinases p38 and JNK phosphorylate ATF-2 at amino acids Thr69 and Thr71 [235,236]. The phosphorylated ATF-2 can then form homodimers and heterodimers [237], which bind with high affinity to the consensus sequence 5`-TGACGTCA-3` [238] in target gene promoters, resulting in their activation. The phosphorylation of ATF-2 thus results in the expression of a broad spectrum of proteins implicated in different

processes, such as cell cycle molecules (cyclin D1) [239], cell adhesion molecules [240], growth factors [241], anti-apoptotic factors [242], and invasion-related molecules [243].

To confirm that PknB is able to phosphorylate ATF-2, we incubated ATF-2 with P knB *in vitro* and performed mass spectrometric analyses. As a positive control we incubated ATF-2 with p38. The results show that PknB can indeed phosphorylate ATF-2 (Fig. 2).

However, PknB phosphorylated Thr73, whereas p38 phosphorylated Thr69 and Thr71. Interestingly, Thr73 is the known phosphorylation site of the Human Vaccinia-related Kinase 1 (VRK1) [244]. VRK1 is a ser/thr kinase overexpressed in proliferating cells [245,246]. Phosphorylation of ATF-2 on Thr 73 by VRK1 kinase leads to the activation of ATF-2 and, consequently, the induction of cellular protection mechanisms.

Another peptide that was very efficiently phosphorylated by PknB belongs to the Bcl-2 interacting protein Bim (see Table S2 available on PLos One website). *In vivo* this peptide is recognized by the c-Jun NH_2-terminal kinase (JNK) (Table S1) [247,248]. Bim is a member of the pro-apoptotic Bcl-2 family of proteins, which play a critical role in apoptosis regulation. A short peptide motif, DKSTQT^{56}P, which is present in BimL and BimEL, but absent from BimS, mediates the binding of Bim to the LC8 cytoplasmic dynein light chain. Importantly, the same motif is also recognized and phosphorylated by PknB (Table S1- see PLos One website). Exposure of cells to stress causes the activation of JNK kinase, which phosphorylates Bim at Thr56 in the afore-mentioned DKSTQTP motif. The phosphorylation leads to conformational changes in Bim and subsequent dissociation of Bim from dynein motor complexes [249]. The activated Bim may directly activate pro-apoptotic Bax, or indirectly activate Bax by binding anti-apoptotic Bcl-2 family proteins (e.g., Bcl-2 and Bcl-X_L) [250,251]. Judged by the observed phosporylation of the DKSTQT^{56}P peptide, the release of PknB from invasive *S. aureus* cells might have similar effects as its phosphorylation by JNK.

Table 1. Cellular processes that can be targeted by PknB. Functional classes of human proteins that are potentially phosphorylated by PknB, as identified in the present PepChip analysis, are classified by their biological function.

Transport **Total: 5 proteins**	**Cell growth and maintanance** **Total 10 proteins**
Membrane transport proteins, Voltage gated channel, Water channel proteins	Structural proteins, Cell cycle regulation proteins, Cell adhesion proteins
Metabolism **Total: 2 proteins**	**Immune response and recognition** **Total: 3 proteins**
Phosphotransferase, Ribosomal subunit	Immunoglobulin, Cell surface receptors
Signal transduction and Cell communication **Total: 32 proteins**	**Regulation of nucleobase, nucleoside, nucleotid and nucleic acid metabolism** **Total: 13 proteins**
Receptors, Cell cycle proteins, Cell junction proteins, Serine/threonine kinases, Tyrosine kinases, Transport/cargo proteins, Adapter molecules	DNA binding site, RNA binding site, Ribonucleoproteins, Transcription factors, Transcription regulatory proteins
Apoptosis **Total: 1 protein**	**Stress** **Total: 2 proteins**
Bcl2- interacting protein BIM	Heat-shock proteins

A third intriguing target of PknB identified through phosphorylation profiling is the cytoskeleton-associated protein paxillin. This protein has previously been reported as a target for the phosphatase YopH, which is injected by *Yersinia* into human host cells, thereby affecting cytoskeleton integrity [252]. Taken together, the identified potential targets of PknB are fully consistent with previously reported effects of *S. aureus* and other bacteria on host cell apoptosis [64,253,254,255] and a wide range of cellular processes [200,202,209,223, 224,256,257].

Interestingly, a comparison of the identified human phosphorylation sites of PknB with staphylococcal proteins revealed about 300 putative PknB target sequences in proteins of *S. aureus* (data not shown). These include amino acid sequences in proteins that are known to be phosphorylated by PknB, such as triosephosphate isomerase, DnaK, elongation factors, ribosomal proteins and trigger factor [104].

PknB is a proline-directed serine/threonine kinase

To determine which amino acids are preferably phosphorylated by PknB, we generated a sequence logo based on the 15% best-phosphorylated peptides (Fig. 3). The most frequently found amino acids in PknB-phosphorylated peptides are serine and threonine. This observation is in agreement with the serine/threonine kinase signature in the primary sequence of PknB. Apparently, the signature has portability across the eukaryotic/prokaryotic divides. Nevertheless tyrosine phosphorylation can also be unambiguously identified making this study the first demonstration of enzymatic tyrosine kinase activity in a single isolated prokaryotic enzyme. What is also clearly evident in the sequence logo is the presence of a proline residue next to phosphorylated serine, threonine or tyrosine residues. Thus, it seems that proline is part of the PknB recognition and target sequence. This links PknB to the evolutionary well-conserved family of proline-directed kinases, which includes cyclin-dependent protein kinases (CDKs), mitogen-activated protein kinases (MAP kinases) and glycogen synthase kinase-3 (GSK-3; Fig. 3). These kinases play a crucial role in cell cycle, transcription, signal transduction and are involved in many diseases like cancer or Alzheimer's disease [258,259,260]. Like the eukaryotic MAP kinases, PknB has the ability to phosphorylate ATF-2 at least *in vitro*, which implies that PknB has a MAP kinase-like enzymatic activity. This view is supported by the observation that PknB is also involved in the regulation of important cellular functions in *S. aureus*, including central metabolic pathways [104] and cell wall metabolism [261]. Taken together, our results imply that PknB is the first prokaryotic representative of the proline-directed kinases, a cardinal family of regulators of eukaryotic cellular physiology. A major challenge for future studies will be to identify human proteins that are phosphorylated by PknB *in vivo*, for example upon internalization of *S. aureus* by macrophages.

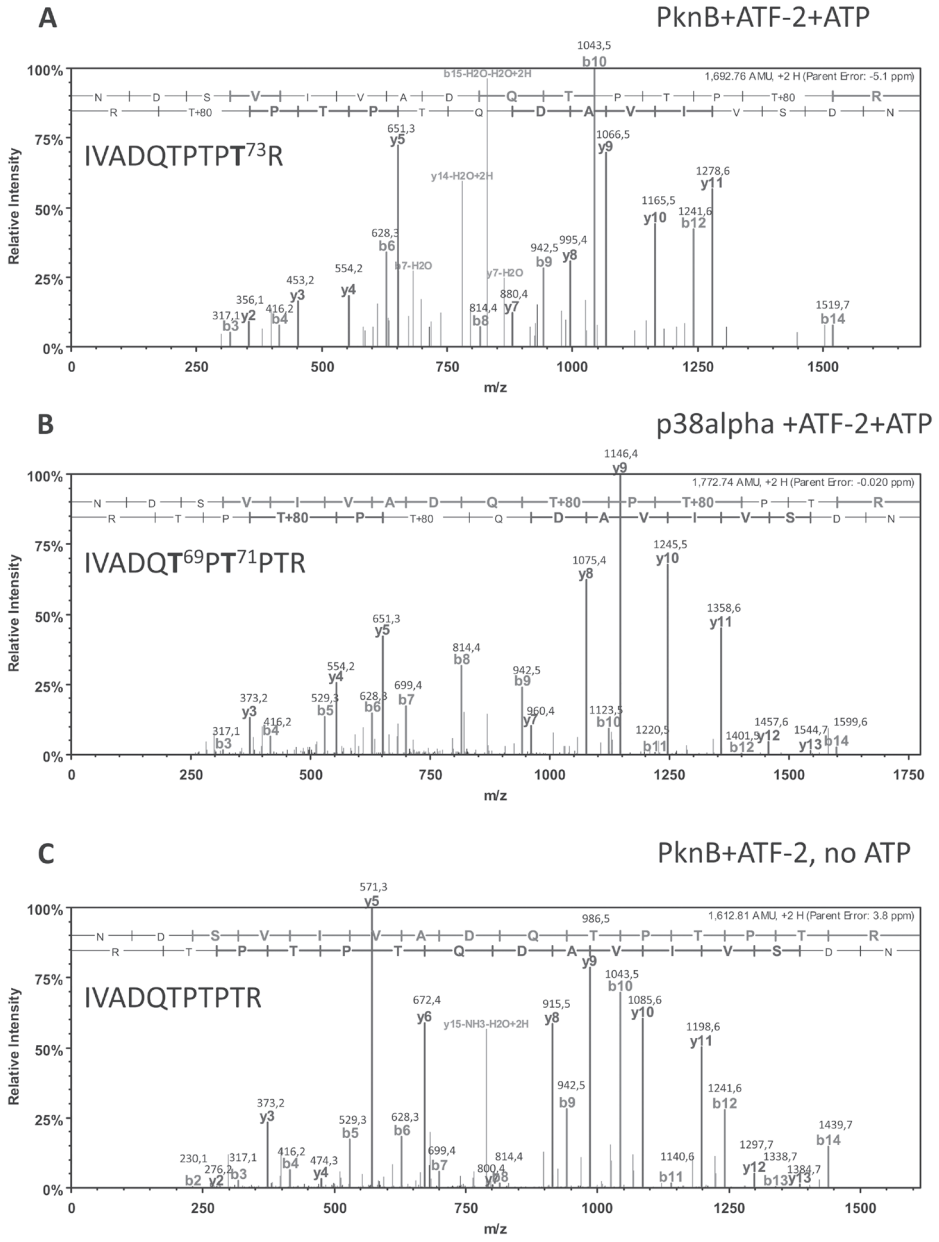

Figure 2. Verification of PknB-dependent phosphorylation of ATF-2. Recombinant ATF-2 was incubated with PknB (A) or p38 (B) in kinase reaction buffer for 30 minutes at 37°C. As a control, ATF-2 was incubated with PknB in the absence of ATP. After tryptic digestion, the resulting peptides were analyzed via online-mass spectrometry. The panels show the spectra for the ATF-2 peptide VIVADQTPTPTR that was either phosphorylated at the Thr73 by PknB, or Thr 69 and Thr 71 by p38. The b- and y-ions are high-lighted and the observed masses are given. Also the peptide sequence is indicated and amino acids that have been identified my mass spectrometric analysis are indicated in bold letters. The upper sequence corresponds to the b- and the lower sequence to the y-ions.

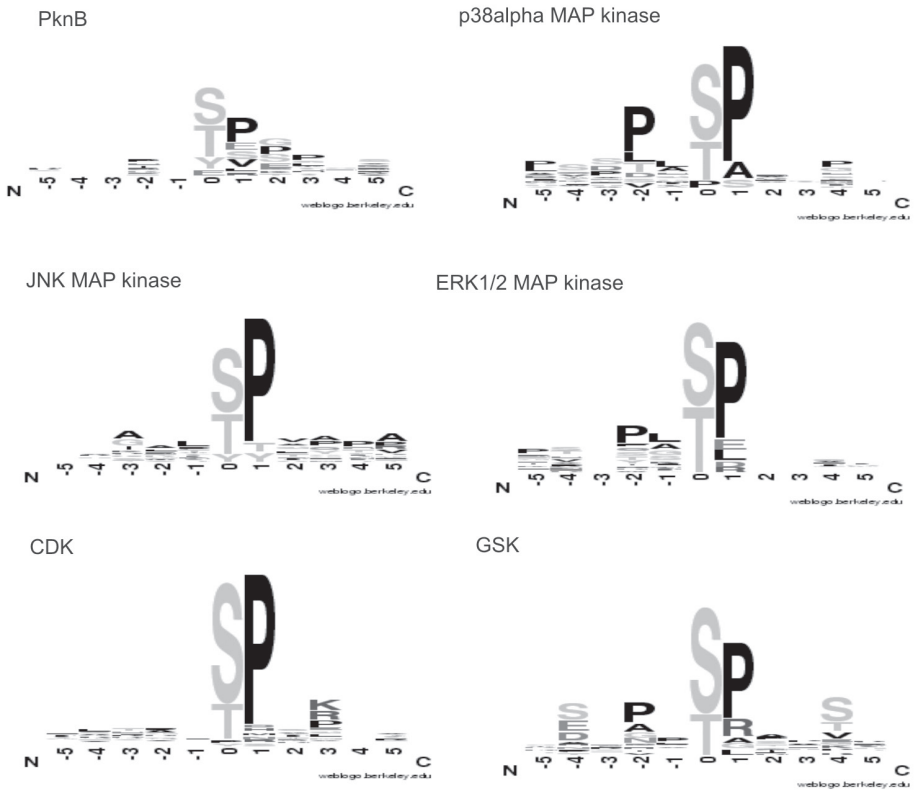

Figure 3. Sequence logo of PknB phosphorylation sites and comparison to known phosphorylation sites of human kinases. The image shows consensus recognition sites for the staphylococcal PknB and other proline-directed ser/thr kinases.

Materials and Methods

Detection of extracellular PknB by immunoblotting

S. aureus NCTC 8325 or a *pknB* mutant of this strain [262] were cultivated in 10 ml of TSB (37 °C, 250 rpm) and growth was monitored by OD_{600} readings. Samples of 3 ml were harvested at OD_{600} 2 and cells were separated from the growth medium by centrifugation (8000 rpm, 5 min, 4 °C). Bacterial cells were washed in PBS, resuspended in sample buffer (NuPage, Invitrogen) and disrupted using a Precellys 24 bead beater (three times 30s, 6800 rpm; Bertin Technologies). Proteins from the growth medium fraction were collected by TCA-acetone precipitation [263]. Protein samples were mixed with gel-loading buffer with reducing agent and incubated for 5 min at 95 °C. To receive a clear signal for the supernatant fractions twice as much as from the crude extract was applied to gel electrophoresis. The proteins were separated on a 10% Bis-Tris gel (Invitrogen) at 200 V for 35 min in NuPAGE® MES SDS Running Buffer (Invitrogen). The separated proteins were transferred to a nitrocellulose membrane (Protran®, Schleicher and Schuell) by semi-dry blotting at 200 mA for 75 min. Membranes were blocked for one hour in Blocking Buffer (Odyssey, Li-Cor biosciences). Rabbit primary antibodies against the kinase domain of PknB were added (1:5000 in blocking buffer) and membranes were incubated for 1 hour. Next, membranes were washed 3 times for 5 minutes in PBS-T (Phosphate Buffered Saline Tween-20) before adding a fluorescent secondary antibody at a 1:20000 dilution in blocking buffer (IRDye 800 CW goat anti-rabbit antibody from LiCor biosciences). Membranes were incubated for 1 hour in the dark, washed three times for 5 min in PBS-T and once in PBS. After transferring the membranes into fresh PBS, they were scanned using the Odyssey Infrared Imaging System (LiCor Biosciences). As a cell lysis control, antibodies against cytoplasmic protein TrxA were used in the same concentration as PknB antibodies.

Cloning, expression and purification of *S. aureus* TrxA

All procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* DH5α cells were carried out as previously described [264]. Genomic DNA of *S. aureus* RN4220 [265] was isolated using the Genelute Bacterial Genomic DNA kit (Sigma). The *trxA* gene on this genomic DNA was PCR-amplified using the primers GGGGGCATATGGCA ATCGTAAAAGTAA and GGGGGCTCGAGTAAATGTTTATCTAAAACTTC. The PCR

product was cloned into *Hinc*II (*Hind*II) restricted pUC18 [266]. After verification of the sequence, the *trxA* gene was excised from this plasmid with *Nde*I and *Xho*I, and ligated into the same restriction sites of pET26b(+) (Novagen, Inc.), downstream of the T7 promoter and upstream of an in-frame His(6)tag sequence. The resulting pET26-SatrxA plasmid was checked by sequencing and used to transform *E. coli* BL21(DE3) (Invitrogen) for high-level *trxA* expression and purification. 10 ml of an overnight culture of this strain was used to inoculate 1 liter fresh LB medium and grown until an OD_{600} of 0.7 was reached. Then, isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM to induce TrxA production. After 3 hrs of induction, cells were harvested by centrifugation and resuspended in binding buffer (20 mM NaPi, 300 mM NaCl, 10 % (v/v) glycerol, 5 mM imidazole, 3 mM DTT, pH 7.4). Next, cells were disrupted by two passages through a French Press (2500 PSI). Cellular debris was removed by centrifugation (30 min at 30000g, 4^{o}C), and the clarified supernatant fraction was applied to a nickel-charged IMAC column (5 ml HisTrap HP, GE Healthcare). Unbound sample was washed from the column with binding buffer using an ÄKTA explorer (GE Healthcare). Next, the His-tagged TrxA protein was eluted from the column using binding buffer with a gradient of increasing imidazole concentrations (up to 500 mM imidazole). The eluted fractions were checked for the presence of pure TrxA protein using SDS-PAGE and subsequent silver-staining. Further purification was achieved by concentrating the proteins with Vivaspin columns (Vivascience) and loading them on a Superdex 75 gel filtration column (Amersham) pre-equilibrated with 20 mM NaPi, 150 mM NaCl, 10% glycerol and 3.5 mM DTT, pH 7.4. Fractions containing the purified TrxA proteins were pooled and dialyzed 3 times against 20 mM Tris-HCl, pH 7.6, with 150 mM NaCl. Specific polyclonal antibodies against the purified TrxA protein were raised in rabbits (Eurogentec).

Kinome array analysis

PepChip™ Kinomics slides containing 976 fully annotated, disease-related kinase phosphorylation sites in triplicate (Pepscan, Lelystad, The Netherlands, http://www.pepscan.com/) were incubated with 50 µl of the ser/thr kinase PknB incubation mix (0.8 µg/ml PknB kinase domain, 60 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 3mM $MnCl_2$, 1 mM DTT, 50% glycerol, 50 µM ATP supplemented with 1Mbq [γ-^{33}P] ATP, 0.03% Brij-35, 50 µg/ml bovine serum albumin, 3 mM Na_3VO_4, 50 µg/ml PEG 8000) for 90 min in a humidified incubator. As a negative control we

used the "empty" incubation mix without PknB. After incubation the peptide arrays were washed twice in 2 M NaCl (1% Tween-20) and PBS-T. Next, the arrays were rinsed twice in demineralised water and air-dried. The dried slides were transferred to a phosphor imager plate (Fuji Storm 860, Stanford, GE, USA) and exposed for 72 hours. The density of the spots was measured and analyzed with array software.

Peptide array data analysis

To analyze the intensity of spots and to correct for background phosphorylation, the ScanAnalyze software and grid tools were used, and the resulting data were exported to an excel sheet. Three replicate data sets were taken for further statistical analysis. To this end, the Spearman correlation coefficient was calculated for each combination of the three sets. The average and standard deviation for each peptide were determined and plotted in an amplitude-based hierarchical fashion. If only background phosphorylation is present, this amplitude-based distribution can be described by a single exponent. Thus, determining the exponent describing amplitude behavior of the 500 least phosphorylated peptides should give an adequate description of array background phosphorylation and, in practice, this was indeed the case. 125 Peptides which exhibited the incorporation of γ-^{33}P in the absence of added kinase were excluded from further analysis. Peptides of which the average phosphorylation minus 1.96 times the standard deviation was higher as the value expected from describing the background distribution were considered to represent true phosphorylation events. Two-sided heteroscedastic t-tests were also performed on each set of values to determine significance ($p<0.05$).

Sequence logos

Sequence logos were created with the weblogo server at http://weblogo.berkeley.edu/logo.cgi using either the preferred substrates of PknB, or known phosphorylation sequences for human kinases as available at http://www.phosphosite.org/homeAction.do

In vitro phosphorylation of ATF-2 by staphylococcal PknB

In order to confirm the phosphorylation of ATF-2 by PknB, the *in vitro* assay was performed. Purified staphylococcal kinase PknB (26 µg) was incubated with 50 µg of Activating Transcription Factor fusion protein (Cell Signaling) in kinase

incubation mix (50 mM HEPES, 1 mM DTT, 0.01 Brij35, 3 mM $MnCl_2$, 3 mM $MgCl_2$, 50 µM ATP) for 30 minutes at 37°C in waterbath. As a positive control, 0.1 µg of p38-alpha MAP kinase (Cell Signaling) was incubated with 50 µg of ATF-2. As negative controls, the following reaction mixtures were used: PknB with ATF-2 but without ATP; PknB with ATP but without ATF-2; and ATF-2 with ATP but without PknB.

Phosphorylation site identification and protein identification by mass spectrometry

Trypsin (Promega) was activated by 15 min incubation at 30 °C in activation buffer and then added 1:200 to the samples, digestion was allowed to proceed over night at 37 °C. The resulting peptides were separated by liquid chromatography and measured online by ESI-mass spectrometry using a nanoACQUITY UPLC™ system (Waters, Milford, MA) coupled to an LTQ Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were desalted onto a trap column (Symmetry® C18, Waters). Elution was performed onto an analytical column (BEH130 C18, Waters) by a binary gradient of buffer A (0.1% (v/v) acetic acid) and B (100 % (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 50 min with a flow rate of 400 nl/ min. The LTQ Orbitrap was operated in data-dependent MS/MS mode using MSA for phospho-relevant masses. Proteins were identified by searching all MS/MS spectra in .dta format against all *S. aureus* NCTC 8325 proteins and added ATF-2 protein (extracted from the NCBI database) using Sorcerer™-SEQUEST® (ThermoFinnigan, San Jose, CA; version v.27, rev. 11). Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10 ppm. Up to two missed tryptic cleavages were allowed. Methionine oxidation (+15.99492 Da), Carbamidomethylation (+57.021465 Da) and phosphorylation of STY (+79.966331 Da) was set as variable modification. Proteins were identified by at least two peptides applying a stringent SEQUEST filter. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.75 and 3.75 for singly, doubly, triply and quadruply charged peptides. Phosphorylated peptides which passed this filter were examined manually and accepted only, when b- or y- ions confirmed the phosphorylation site.

Acknowledgements

The authors wish to thank Michel Débabouillé and Tarek Msadek for useful discussions. M.M. was supported by an Ubbo Emmius fellowship from the University of Groningen. J.M.v.D., M.P.P. and K.O. were in parts supported by the CEU projects LSHM-CT-2006-019064 and LSHG-CT-2006-037469, the transnational SysMO initiative through project BACELL SysMO, the European Science Foundation under the EUROCORES Programme EuroSCOPE, grant 04-EScope 01-011 from the Research Council for Earth and Life Sciences of the Netherlands Organization for Scientific Research, and Top Institute Pharma project T4-213. K.O., S.D. S.R. and T.S. were supported by a grant of the Deutsche Forschungsgemeinschaft (TR34).

Chapter 4

The staphylococcal kinase PknB alters tyrosine kinase signaling in macrophages and attenuates host responses

Submitted to the *Journal of Proteome Research*

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Abstract

Infections caused by *Staphylococcus aureus* represent a serious health problem often associated with fulminant inflammatory responses. Detailed mechanistic insights how *S. aureus* infections provoke inflammation are urgently needed to devise novel therapeutic approaches. Here we report the function of the prokaryotic-type serine/threonine kinase PknB of *S. aureus* in inflammatory responses of human immune cells. We show that stimulation of human peripheral blood monocytes with PknB-proficient *S. aureus* provokes a strong pro-inflammatory cytokine production that is largely absent when these cells are stimulated with *S. aureus* lacking PknB. Furthermore, kinase profiling of host responses using peptide arrays reveals that PknB-proficient *S. aureus* provokes markedly stronger kinase activation upon macrophage infection than *S. aureus* lacking PknB. Specifically, stimulation with PknB-deficient *S. aureus* results in a significant down-regulation of tyrosine kinases, like Bruton's kinase, that are known to be crucial for Toll-like Receptor 2-mediated signaling and cytokine production. In conclusion, *S. aureus* requires PknB to provoke strong inflammatory responses, and specific PknB inhibitors may therefore find future applications in combating the fulminant pathology of *S. aureus* infection.

Introduction

S. aureus is an opportunistic pathogen that is carried by about 30% of the human population. While carriage of this Gram-positive bacterium is of no serious consequence to most individuals, *S. aureus* has the potential to cause a wide range of infections ranging from minor skin infections to more serious invasive diseases, such as bacteremia, sepsis, abscesses of various organs, osteomyelitis, endocarditis, pneumonia, and meningitis [2,3,4]. Sepsis is caused by the widespread activation of immune cells resulting in the release of an array of inflammatory mediators, such as cytokines, chemokines, prostaglandins and lipid mediators, as well as reactive oxygen species. This can provoke a life-threatening cytokine storm and multi-organ failure, mostly involving the lungs, liver and kidneys [267]. Mechanistic insights into the factors that mediate the unusual propensity of *S. aureus* to provoke fulminant sepsis as compared to other bacterial species is urgently needed to develop rational therapeutic avenues to improve disease outcome.

The strong reaction to *S. aureus* bacteraemia is largely due to the presence of immunostimulatory components on the surface of the microorganism. In fact, about 50% of the staphylococcal cell is composed of peptidoglycan (PepG) and other pro-inflammatory molecules, which are released upon bacterial lysis or may be detected by the immune system in the bacterial context. Upon binding of cell wall components to pattern recognition receptors of host immune cells, specific signal transduction mechanisms are enkindled that culminate in pro-inflammatory gene transcription. Altered phosphorylation of proteins is a key element in the intracellular propagation of the pro-inflammatory signal. Thus, phosphorylation patterns are exquisitely sensitive to changes in pro-inflammatory stimulation of immune cells and their study by generating comprehensive descriptions of cellular signaling using peptide arrays is exceedingly useful for the mechanistic dissection of pathogenic responses.

Interestingly, in recent years it has emerged that also in prokaryotes phosphorylation is an important regulatory mechanism. In prokaryotes, signal transduction often involves so called two-component regulatory systems, which are composed of a histidine kinase and a response regulator. However, recent studies have shown that eukaryotic-like serine/threonine protein kinases (STPKs) are also expressed by many prokaryotes for the regulation of various cellular functions, such as stress responses, biofilm formation, sporulation, metabolism

and developmental processes [98,99,100,101,102]. STPKs also play critical roles in the virulence of many pathogens, such as *Yersinia pseudotuberculosis*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* [206,207], *Streptococcus pneumoniae* [208,209,210] and *Staphylococcus aureus* [103,104,106]. However, in many cases the precise biological functions and roles that these kinases might play in infection have remained enigmatic.

The staphylococcal STPK, often referred to as PknB, is composed of three extracellular PASTA domains (penicillin-binding domains), a central transmembrane domain and an intracellular kinase domain [103]. Interestingly, it was recently reported that PknB is not only involved in the regulation of various metabolic pathways and cell wall synthesis of *S. aureus* [102,104], but that it also determines the infectivity of *S. aureus* in a mouse kidney abscess model [106]. The latter observation raises the question whether the kinase activity of PknB is directly or indirectly involved in the pathogenicity of *S. aureus*. A direct role for PknB is conceivable, because we observed full-size PknB in the growth medium of *S. aureus* [196]. Subsequent analyses revealed that PknB can phosphorylate specific human target proteins. In fact, the results indicated potential roles for extracellular PknB in a wide range of host cell signalling processes during *S. aureus* infection [196]. A direct role of PknB in infection is also conceivable since serine/threonine kinases have been shown to play key roles in mammalian cell signaling, and at least two bacterial equivalents, YpkA of *Yersinia* and PknG of *Mycobacterium tuberculosis*, are known to be directly involved in the subversion of host cells during the respective infectious processes [223,224]. However the exact role played by PknB in the pathogenesis or persistence of *S. aureus* and especially its importance in the unusually fulminant immune responses to this microorganism remains to be defined.

In the present studies, we investigated the roles of *S. aureus* PknB in infection of human peripheral blood mononuclear cells (PBMCs) and THP-1 macrophages using enzyme-linked immuno sorbent assays (ELISA) and kinase profiling. The results show that infection of these cells with an *S. aureus pknB* mutant (Δ*pknb*) results in reduced secretion of inflammatory mediators and reduced activity of important pro-inflammatory kinases, such as the Bruton's tyrosine kinase, focal adhesion kinases and Src kinases. Since kinases are highly

druggable, we propose that pharmacological inhibitors of PknB may be useful therapeutics for combating an *S. aureus* infection-dependent cytokine storm.

Results and Discussion

Limited cytokine production by PBMCs upon stimulation with *pknB* mutant *S. aureus*

In order to study the effect of PknB on cytokine production by PBMCs, we incubated PBMCs from healthy donors for 1, 4, 8 and 24 h at different multiplicity of infection (MOI) levels with cells of an *S. aureus pknB* mutant or the parental strain NCTC 8325 (Fig. 1 and Supplementary Fig. 1). The *S. aureus* strain NCTC 8325 and its *pknB* mutant derivative were selected for these studies, because these strains have been used in many previous studies on staphylococcal virulence and PknB function [1,103]. We observed that PBMCs incubated with the *pknB* mutant strain produced dramatically less inflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-10 and TNFα, than cells infected with the parental strain at all MOIs (Fig. 1 and Supplementary Fig. 1). Thus, the genetic absence of PknB remarkably reduces the pathogenic nature of *S. aureus*.

Staphylococcal PknB affects the activity of protein tyrosine kinases and PI3 kinase in THP-1 macrophages

Our cytokine secretion experiments revealed a significantly lower production of inflammatory cytokines when PBMCs were incubated with *pknB* mutant cells. Therefore, we investigated whether these changes can be correlated to reduced activity of host kinases using kinase profiling as previously described [268]. The advantage of this experimental approach is that it allows the investigation of early responses to *S. aureus* that precede the actual production and secretion of cytokines. Accordingly, human THP-1 macrophages were incubated for 15 min, 1 h or 24 h either with *S. aureus* Δ*pknb* or with the parental strain NCTC8325, and the responses within the THP-1 macrophages were assessed by kinase profiling. This revealed a number of peptides that are substrates of kinases of which the activities are significantly different upon incubation with *S. aureus* NCTC 8325 or the *pknB* mutant (Fig. 2). Supplemental Table 1 lists the peptide substrates that were phosphorylated to significantly different extents when THP-1 macrophages were incubated with the *pknB* mutant for 15 min, 1 h or 24 h.

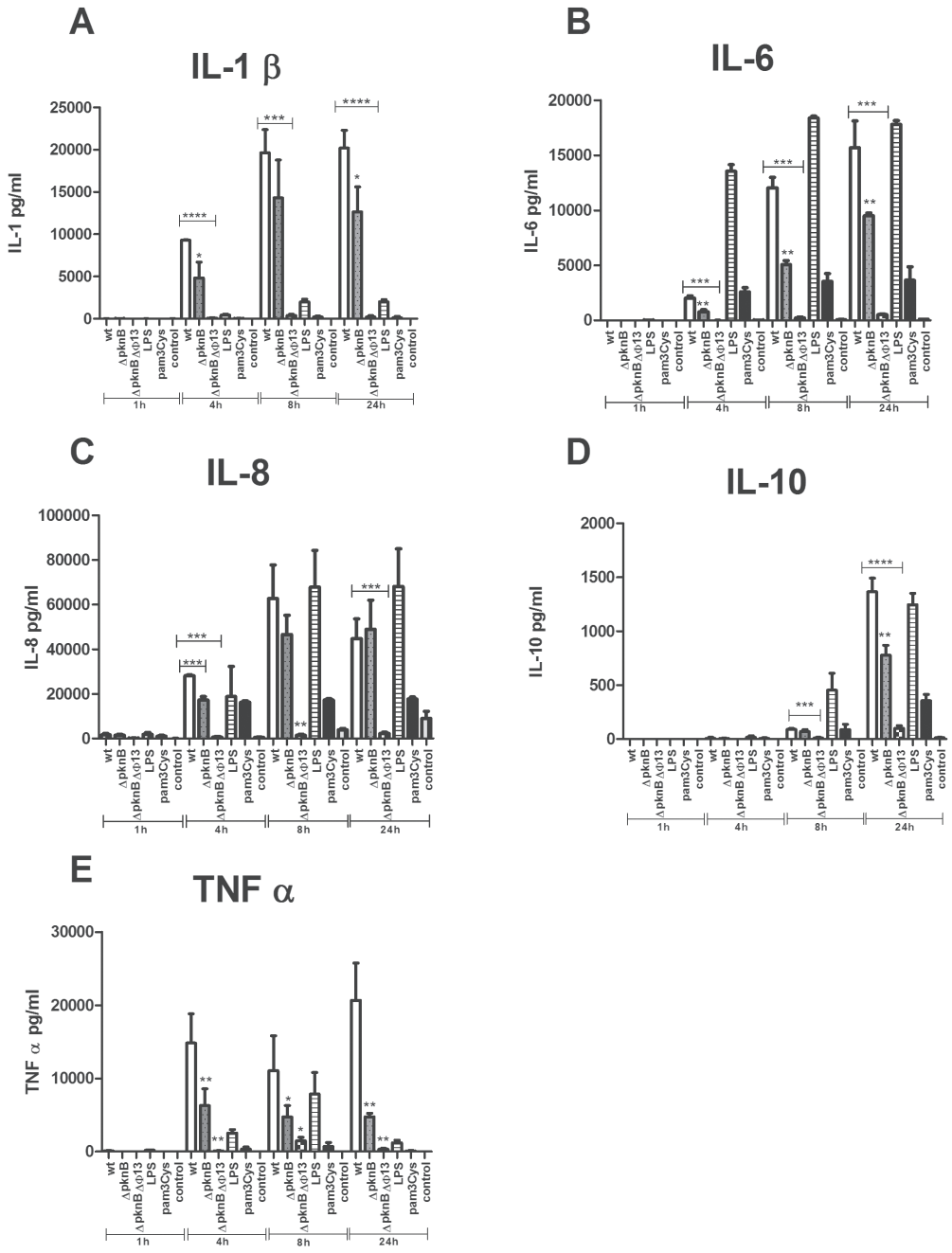

Figure 1. Cytokine production by PBMCs incubated with S. aureus. Average concentrations of IL-1β (A), IL-6 (B), IL-8 (C), IL-10 (D) or TNF-α (E) secreted by PBMCs incubated with S. aureus strains NCTC 8325 (wt), ΔpknB or ΔpknBΔϕ13 for 1 h, 4 h, 8 h or 24 h are indicated. *P<0.05, ** P<0.01, *** P<0.001 for each cytokine response among the three strains as determined by Student's t-tests.

Overviews of the kinases and signaling pathways in THP-1 macrophages that were differentially influenced upon incubation with the tested *S. aureus* strains are presented in Figures 3 and 4, and the respective signaling pathways are schematically represented in Figure 5. Overall, the results showed that at 15 min post infection, compared to the parental strain, the *pknB* mutant increased the levels of Receptor Tyrosine Kinase signaling and apoptotic signaling, whereas Ca^{2+} signaling and stress responses were less pronounced (Fig. 3 and 4).

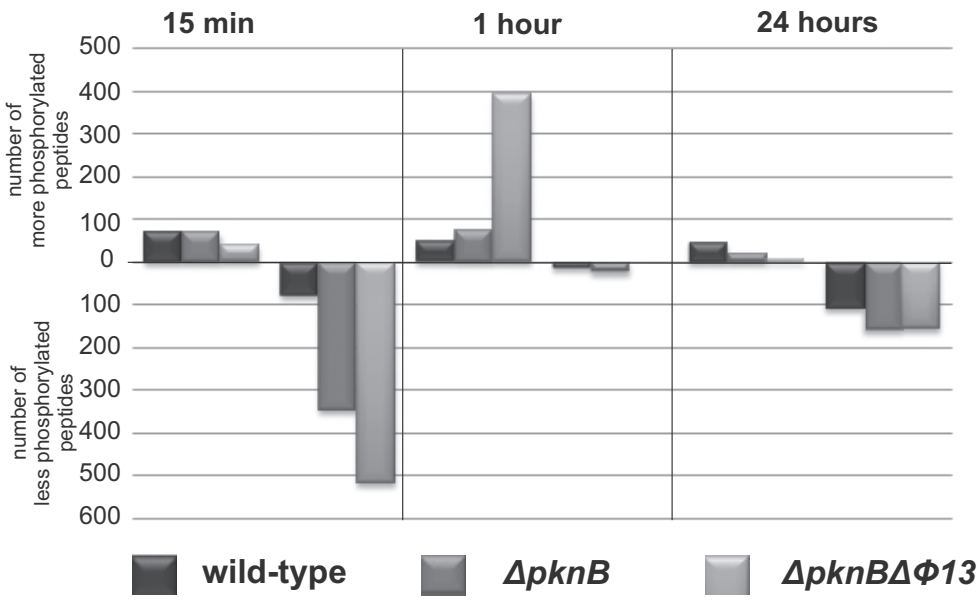

Figure 2. Overview of kinase profiling results. The diagram shows the numbers of differentially phosphorylated peptides upon incubation of pepchips with lysates of THP-1 macrophages incubated with different *S. aureus* strains (as indicated), or with THP-1 macrophages that were incubated without *S. aureus*.

At 1 h post infection, the *pknB* mutant triggered increased levels of PKC kinase signaling, Ca^{2+} signaling and apoptotic signaling, whereas the levels of cell cycle signaling, the cAMP-dependent signaling and MAPK signaling were reduced. However, it should be noted that the changes in the levels of kinase activities at 1 h post infection were much smaller than at 15 min post infection, and also the differences in the responses to the *pknB* mutant or the parental strain were much smaller at this time point (Fig. 3A). At 24 h post infection, there was no major

difference detectable in the THP-1 signaling pathways that were modulated in response to *S. aureus* NCTC 8325 or the *pknB* mutant (Fig. 4). Nevertheless, significant differences for individual kinases were still detectable at 24 hr post infection (Fig. 3A).

Specifically, the incubation of THP-1 macrophages with *S. aureus* Δ*pknb* for 15 min resulted in the activation of known signaling pathways for early phagocytosis and cytoskeletal rearrangement. During the first 15 min post infection with the *pknb* mutant, the activity of 22 out of 36 kinases was reduced as compared to the control macrophages that were not incubated with *S. aureus* (Supplemental Table 1). The strongest decreases (0.64 fold) were observed in the activity of the Bruton's kinase (Btk), Focal adhesion kinase (FAK) and Src kinase (Fig. 3A). By contrast, FAK and Src were slightly upregulated, and Btk was slightly downregulated when THP-1 macrophages were incubated with the parental *S. aureus* strain (Fig. 3A) [1]. Moreover, kinases like PAK and PI3K were differentially regulated when macrophages were incubated with *S. aureus* Δ*pknb* or the parental strain (Figs. 3A and 4). Bruton's kinase belongs to the Tec family of cytoplasmic receptor tyrosine kinases and is mainly expressed in B cells and in myeloid cells, such as macrophages and neutrophils [269]. Btk deficiency results in the human disease called X-linked agammaglobulinemia (XLA). Patients with XLA suffer from recurrent bacterial infections due to reduced numbers of mature B cells [270]. Like the SFK kinases, the FAK kinase showed significantly decreased activity (0.56-fold) upon infection of THP-1 macrophages with *S. aureus* Δ*pknb*. The protein tyrosine kinase FAK is recruited at an early stage to focal adhesions and it mediates many of the downstream responses, such as phagocytosis. FAK is activated by the focal adhesion complex (FAC) and by binding of β subunits of integrins to the extracellular matrix (ECM).

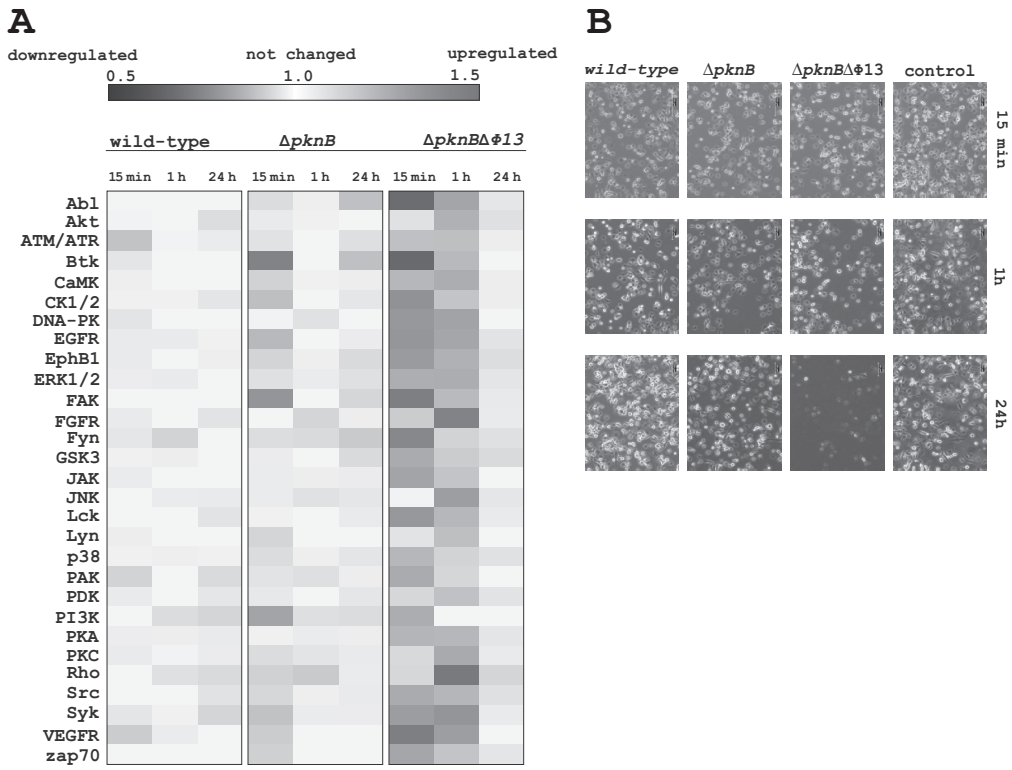

Figure 3. Activity of different kinases in THP-1 macrophages incubated with *pknB* mutant *S. aureus* cells. THP-1 macrophages were incubated with cells of the *S. aureus* strains Δ*pknb* or Δ*pknBΔφ13* or the parental strain NCTC 8325 (wild-type) for 15 min, 1 h or 24 h. As a control, macrophages were incubated without *S. aureus* cells. Kinase profiling and data analyses were performed as described in the Materials and Methods section. (A) Up- or down-regulation of kinase activities in THP-1 macrophages incubated with *S. aureus* NCTC 8325, Δ*pknB* or Δ*pknbΔφ13*. A fold change of 1 represents the situation where the phosphorylation of respective peptides is identical in THP-1 cells incubated with or without *S. aureus*. A fold change of > 1 indicates that the phosphorylation intensity of particular peptides was increased when THP-1 cells were incubated with *S. aureus*, and a fold change <1 indicates that the phosphorylation intensity of particular peptides was decreased when THP-1 cells were incubated with *S. aureus*. Calculated values of the presented up- or down-regulated kinase activities are based upon the data in Supplemental Tables 1 and 2. Note that we used the control data set for the parental strain in the previously published paper by [1]. 2011. The parental control data were however collected in parallel with the data sets for the Δ*pknB* or Δ*pknbΔφ13* strains. (B) THP-1 macrophages incubated with *S. aureus* NCTC 8325 (wt), Δ*pknB* or Δ*pknBΔφ13* for 15 min, 1h or 24 h. Magnification 10x.

Focal adhesion complex assembly and disassembly are critical for cell attachment and movement. When FAK is activated it autophosphorylates and binds the Src kinase (Fig. 5), which in turn phosphorylates other sites on FAK and the FAK-binding proteins, such as Cas and paxillin. Phosphorylation of FAK is also important for the recruitment of other SH2-containing proteins, including phosphotidylinositol 3-kinase (PI3K) and phospholipase-Cγ (PLCγ; Fig. 5). Our present results show that both FAK and PI3K were down-regulated upon THP-1

macrophage incubation with *S. aureus* Δ*pknB* (Fig. 3A). Interference with activity of the FAK-Src complex, which seems down-regulated in response to *S. aureus* Δ*pknB* (Figs. 3A and 5), is expected to impair cell motility in multiple cell types as both enzymes are critical for proper focal contact turnover. Agerer and colleagues have shown that integrin engagement by *S. aureus* triggered recruitment of the focal contact-associated proteins vinculin, tensin and zyxin, and FAK-deficient cells were severely impaired in their ability to internalize *S. aureus* [271]. The results of kinase profiling were verified by Western blotting analyses with phosphoprotein-specific antibodies. Generally, the results of these two types of analyses were in good agreement (Figs 3 and 6). It was however noteworthy that Western blotting revealed strong effects of the *pknB* mutation in *S. aureus* on signaling in the macrophages after 24 h incubations, where strongly lowered levels of the phosphorylated forms of p38, JNK, ERK1/2, IKK-β, Src and paxillin were detectable (Fig. 6).

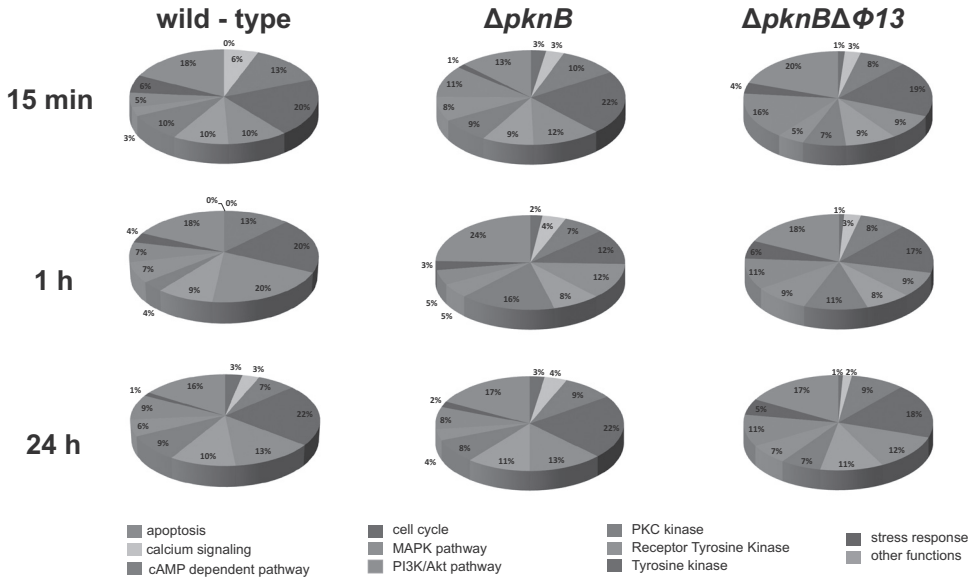

Figure 4. Overview of kinases and signaling pathways that are influenced upon incubation of THP-1 cells with the S. aureus strains NCTC 8325 (wild-type), Δ*pknB* or Δ*pknB*Δ*φ13*. The activity of different kinases and phosphorylation pathways in THP-1 macrophages incubated with *S. aureus* were determined as described in Figure 3. THP-1 macrophages were incubated with *S. aureus* cells for 15 min, 1 h or 24 h. As a control, macrophages were incubated without *S. aureus* cells. Note that we used the control data set for the parental strain in the previously published paper by [1]. 2011. The parental control data were, however, collected in parallel with the data sets for the Δ*pknB* or Δ*pknbΔφ13* strains.

Our findings concerning the cytokine production by *S. aureus*-stimulated PBMCs can best be reconciled with the data obtained for *S. aureus*-stimulated THP-1 macrophages at 15 min post infection. At this time point, we observed a strong down-regulation of Btk when the THP-1 cells were stimulated with *pknB* mutant *S. aureus* cells. This is expected to result in reduced cytokine production. On the other hand the activity of Btk in THP-1 macrophages was enhanced at 1 h post infection with *S. aureus* cells lacking *pknB*. At this time point, the PBMCs did not yet produce detectable amounts of most tested cytokines, not even when they were stimulated with lipopolysaccharide (LPS) or the lipopeptide Pam_3Cys. This makes a direct comparison of the results obtained with THP-1 macrophages and PBMCs a bit difficult, but it is clear that *pknB* mutant cells have a lower propensity to stimulate cytokine production than the parental strain NCTC 8325.

Staphylococcal phage 13 limits the intensity of macrophage signal transduction responses to *pknB* mutant *S. aureus* cells

In the course of our studies we noticed that some of our *pknB* mutant isolates formed an additional halo on blood agar plates. Subsequent analyses by PCR (Fig. 7) and proteomics (data not shown) revealed that these isolates had lost the phage 13. This caused the secretion of active β-hemolysin and, accordingly, a second halo on blood agar. When PBMCs from healthy donors were incubated with the *pknB* mutant strain lacking phage 13 (Δ*pknB*ΔΦ13), they produced barely detectable amounts of IL-1β, IL-6, IL-8, IL-10 and TNFα. (Fig. 1 and Supplemental Fig. 1). Thus, the absence of phage 13 from the *pknB* mutant strain reduced the inflammatory responses to *S. aureus* even further than the *pknB* mutation alone. On the other hand, infection of THP-1 macrophages with *pknB* mutant *S. aureus* cells lacking phage 13 caused severe macrophage lysis upon 24 h incubation (Fig. 3B). Therefore, we decided to also investigate the THP-1 responses to *pknB* mutant cells lacking phage 13 by kinase profiling. Supplemental Table 2 lists the peptide substrates that were phosphorylated to significantly different extents when THP-1 macrophages were incubated with *S. aureus* Δ*pknB* lacking phage 13 or the parental strain for 15 min, 1 h or 24 h. The kinase profile of macrophages incubated with *S. aureus* Δ*pknb* lacking phage 13 was, in essence, comparable to the profile of macrophages infected with *S. aureus* Δ*pknb* containing phage 13 (Fig. 3A). However, especially 15 min and 1 h post infection, the level of apoptotic signaling was higher in THP-1 macrophages incubated with the *pknB* mutant lacking phage 13 (Fig. 4). An intriguing observation was that nearly all THP-1

responses observed for the *pknB* mutant were enhanced upon infection with the strain lacking phage 13, the activity of kinases being more strongly inhibited at 15 min post infection and more strongly induced at 1 h post infection (Figs. 2 and 3A). Such a strong inhibition of kinase activity at 15 min post infection would be consistent with the absence of the phage 13-encoded SCIN, CHIP and SAK proteins that are involved in complement- and inflammatory cell recruitment inhibition [27,31,32,35]. By contrast, the increased activity of kinases at 1 h post infection can be attributed to the detrimental effects of secreted β-hemolysin. This hemolysin is a secreted sphingomyelinase that damages membranes by hydrolyzing sphingomyelin, the most prevalent sphingolipid in the mammalian cell membrane.

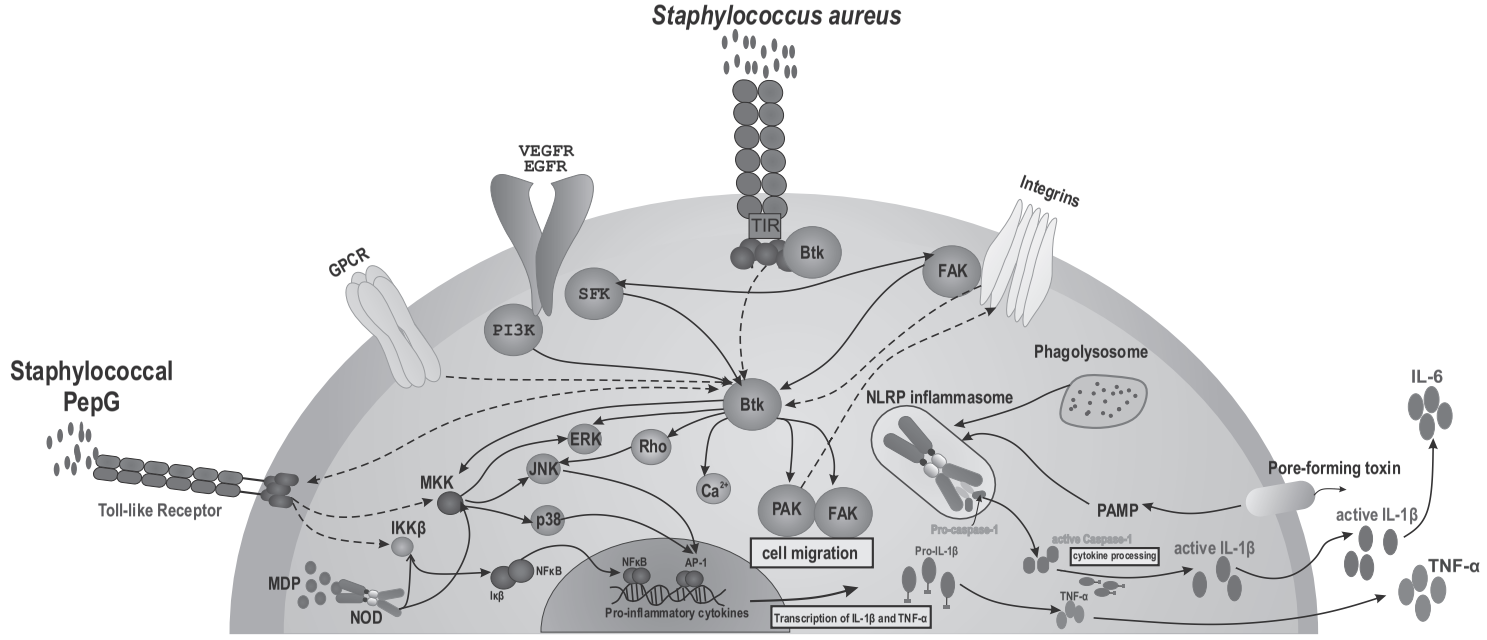

Figure 5. General overview of the signaling pathways active upon incubation of THP-1 cells with *S. aureus* lacking PknB. The kinase profiling results indicate that during incubation with *S. aureus* Δ*pknB* the activities of several kinases are down-regulated, especially the Focal Adhesion Kinase (FAK), Src kinase, Bruton's kinase (Btk) and phosphatidylinositol-3-kinases (PI3K). Kinases that are down-regulated are indicated in green, kinases that are up-regulated are indicated in red, and kinases that are possibly involved in signaling are indicated in yellow. Direct stimulations are indicated by arrows, and tentative stimulatory effects are indicated by dashed arrows. MDP, muramyl dipeptide; MKK, mitogen-activated protein kinase; PAMP, pathogen-associated molecular patterns. All other abbreviations are defined in the main text.

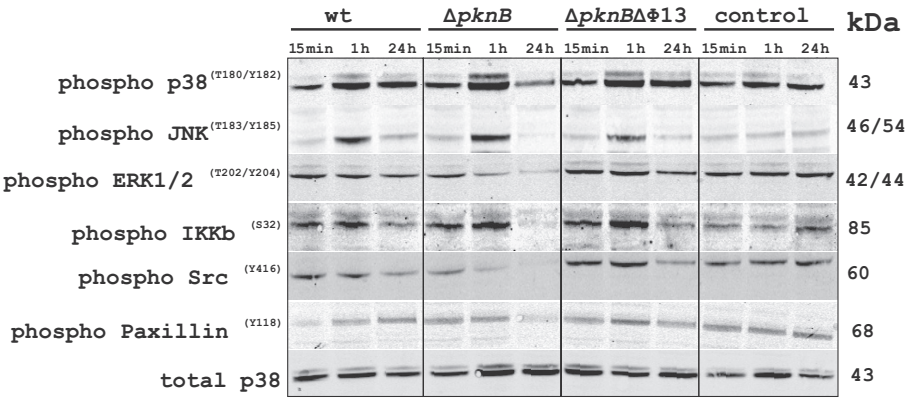

Figure 6. Western blotting controls for *S. aureus*-**induced changes in peptide phosphorylation.** THP-1 macrophages were incubated for 15 min, 1 h or 24 h with *S. aureus* strains NCTC 8325 (wt), Δ*pknB* or Δ*pknBΔφ13*. As a control THP-1 macrophages were incubated in the absence of *S. aureus*. All THP-1 lysates used for Western blotting relate to the same samples that were used for kinase profiling. The membranes were probed with antibodies that are specific for the phosphorylation state of the respective proteins. The apparent molecular weight of each protein is indicated.

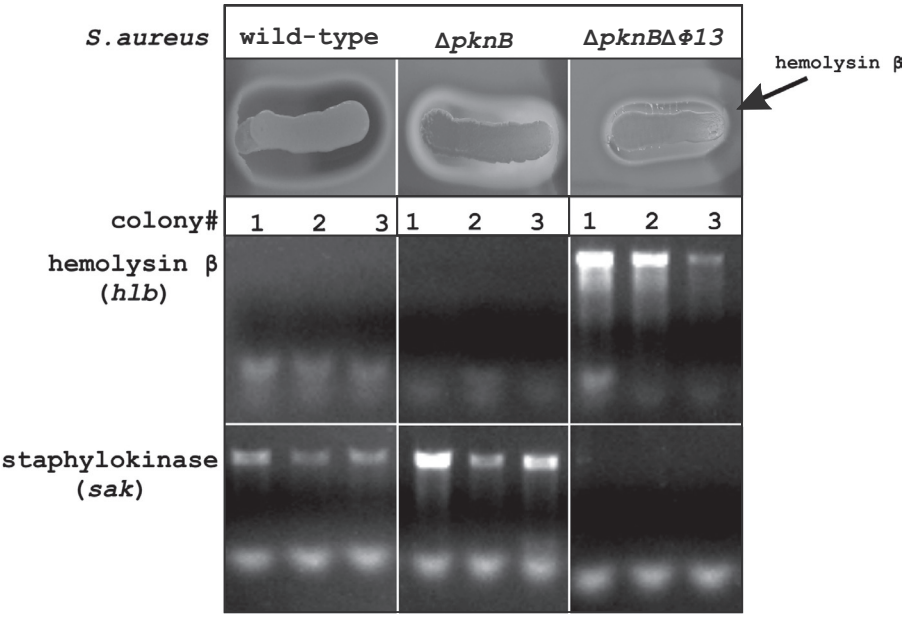

Figure 7. Production of βhemolysin. Upper panel, Colonies of *S. aureus* NCTC 8325 (wt), Δ*pknB* or Δ*pknBΔφ13* (from left to right) grown on blood agar. Note that the colony of *S. aureus* Δ*pknBΔφ13* forms a faint second halo due to the secretion of active hemolysinβ . Middle panel, PCR detection of the intact hlb gene in three isolates of each strain. Lower panel, PCR detection of the phage 13-encoded sak gene in three isolates of each strain. Strains NCTC 8325 and Δ*pknB* show the typical hlb and sak PCR amplification patterns of strains harboring the lysogenic HIb-converting phage 13. In contrast, PCR reveals the presence of an intact hlb gene in the phage 13-cured strain Δ*pknBΔφ13*.

The products of this hydrolytic reaction are phosphorylcholine and ceramide. A variety of mammalian cells was shown to be damaged by β-hemolysin and many diseases have been associated with this toxin [272,273,274,275]. It has been shown that ceramides are involved in the induction of cell death during infection of endothelial cells with *S. aureus.* Nevertheless, the molecular mechanisms by which ceramide and sphingomyelinase are involved in *S. aureus* infections have so far remained unknown [276,277]. Interestingly, it has been also reported that secreted β-hemolysin is required for the induction of caspase-1 and the Nlrp3 inflammasome, as well as cytokine 1 secretion in macrophages incubated with *S. aureus* [94]. Thus, the observed kinase up-regulation at 1 h post infection might be the result of inflammasome activation by β-hemolysin. Clearly, we observed high levels of THP-1 macrophage lysis at 24 h post infection (Fig. 3B), which is consistent with the known cytolytic properties of β-hemolysin. Notably, comparable kinase activities were observed in macrophages incubated for 24 h with *pknB* mutant *S. aureus* with or without phage 13 (Figs. 3A, 4 and 8). This apparent lack of effect, combined with the observed macrophage lysis upon infection with β-hemolysin-producing cells, suggests that the macrophages that had responded strongly to infection with *S. aureus* Δ*pknB*ΔΦ13 at 15 min and 1 h post infection had lysed after 24 h and that only the non-responding macrophages survived until this time point.

We can only speculate about the reasons why cytokine production was close-to-absent upon PBMC incubation with the Δ*pknB*ΔΦ13 strain (Fig. 8). In view of the fact that cells of this *S. aureus* strain do not express SAK, SCIN and CHIPS, but secrete β-hemolysin instead we would have expected a high level of cytokine production, especially because Walev and colleagues have shown that the purified β-hemolysin causes massive cytokine production [278]. However, we did not stimulate the PBMCs with purified β-hemolysin, but with a β-hemolysin-producing *S. aureus* strain. Thus, it is conceivable that at 4 h post infection the β-hemolysin levels were high enough to affect the viability of the PBMCs to the extent that they could no longer produce cytokines. In this respect, it is unfortunate that PBMCs stimulated for 1 h with *S. aureus* cells, LPS or Pam3Cys did not yet start to produce cytokines so that early effects of the Δ*pknB*ΔΦ13 strain on the PBMCs remain obscure.

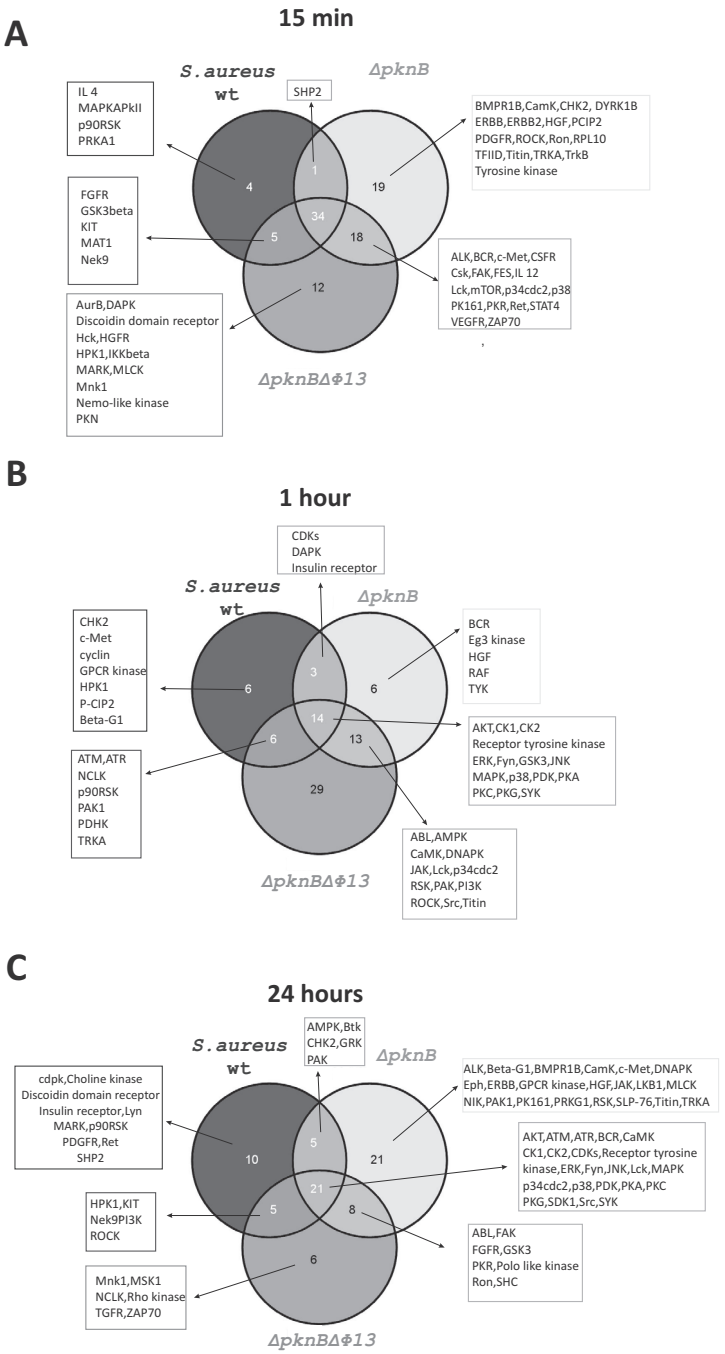

Figure 8. Upstream kinases of which the activity is differentially regulated upon incubation of THP-1 macrophages with or without *S. aureus*. (A) 15 min incubation, (B) 1 h incubation or (C) 24 h incubation. Blue, *S. aureus* NCTC 8325; yellow, *S. aureus* Δ*pknB*; green, *S. aureus* Δ*pknB*Δ*φ13*.

Interestingly, a recent study of Verkaik *et. al* [279] revealed that *S. aureus* NCTC 8325 containing phage 13 was eliminated significantly faster than the strain lacking phage 13 in nasal colonization experiments with healthy human volunteers.

This suggests that during the early stages of colonization, it may be more advantageous for *S. aureus* to produce the hemolysin β for the killing of phagocytes than to produce immune evasion factors encoded by phage 13 (i.e. SAK, SCIN and CHIPS). However, the large majority of *S. aureus* strains isolated from persistent carriers contained phage 13, which suggest that it is more advantageous for *S. aureus* to acquire this phage for persistent nasal colonization [279].

Association of the absence of staphylococcal PknB with reduced macrophage and PBMCs responses

PknB is the only known serine/threonine kinase of *S. aureus* that binds to peptidoglycan (PepG) through extracellular domains, denoted as PASTA domains, and that plays a role in cell wall metabolism. Although the physiological relevance of Ser/Thr phosphorylation/dephosphorylation has been demonstrated by several groups, many basic questions remain unanswered. For example the mechanisms of sensing extracellular and intracellular signals, as well as the correlation of the presence of PknB with *S. aureus* virulence. Therefore we would like to propose three possible scenarios, which might explain the observed reduced cytokine production and down-regulation of kinases in PBMCs and macrophages when incubated with *pknB* mutant *S. aureus* cells. In the first place, secreted PknB from *S. aureus* cells that have been internalized by macrophages might directly impact on tyrosine kinase signaling in the macrophages as was previously proposed [196]. Secondly, the role of PknB in modulating host cell responses can be explained through its role in the regulation of autolysins, which are PepG hydrolases that play important roles in bacterial cell wall turnover, cell division and cell separation. However, PepG is needed to maintain bacterial cell integrity and viability. Therefore it is critically important to maintain a proper balance between new synthesis of PepG and its degradation. Donat *et al.* have shown a 2.2-fold decreased expression of the major *S. aureus* autolysin gene *atl* and a 2.3- to 2.8-fold induction of two autolysis regulators (*fmtA* and *lytR)* and two murein hydrolase gene regulators (*lrgA* and *lrgB*) in *pknB* mutant *S. aureus* cells.

In addition *pknB* mutant *S. aureus* strains produce less UDP-N-acetylmuramates, which are the main precursors for PepG synthesis and they are more resistant to Triton X-100-induced autolysis. Furthermore, studies of Tamber *et al.* have shown that the autolytic activity of *pknB* mutant cells was restored upon addition of the protease inhibitor phenylmethanesulfonylfluoride (PMSF) to the culture medium, which suggest that the autolysin defect of these cells may be due to enhanced serine protease activity [102,103,105]. The PepG release during *S. aureus* autolysis seems to have a very important role because it activates PknB and stimulates bacterial growth and revival from latency [98,280,281,282]. Therefore, we speculate that reduced cell wall turnover in the *pknB* mutant strain can cause lower amounts of peptidoglycan released from the bacteria. In turn, this might affect the recognition of bacterial pepdidoglycan or whole bacteria by the THP-1 macrophages. Reduced activation of recognition receptors would then lead to lower activity of the kinases involved in pro-inflammatory signal transduction and, eventually, to reduced amounts of secreted cytokines. A third explanation for the reduced response of human macrophages during infection with *S. aureus pknB* mutant cells might be the possible occurrence of small structural change(s) in PepG, which could affect the PepG-lipoteichoid acid interactions [105]. Several studies have shown that LTA and PepG interaction is required for cytokine production, septic shock and multiple organ failure, but the mechanisms by which LTA and PepG act in synergy are not clear [283,284]. Studies of Kengatharan *et al.* showed, that a specific fragment of PepG (N-acetylglucosamineb-[1→4]-N-acetylmuramyl-l-alanine–d-isoglutamine) is responsible for the synergism with LTA to induce NO formation in the murine macrophage cell line J774.2. Furthermore, they found that the structure of LTA determines the ability of a particular bacterium to cause shock and multiple organ failure, while PepG acts to amplify any response induced by LTA [30]. However, the details of structural components within LTA and/or PepG, which are essential for the interaction of these two wall fragments have so far remained unknown. In any case, it is conceivable that minor changes in the peptidoglycan or LTA of the *pknB* mutant cells can result in reduced recognition by the host cell receptors which, in turn, might lead to the observed down-regulation of signal transduction pathways and reduced production of pro- and anti-inflammatory cytokines.

Notably, the three possible mechanisms that might explain the observed host cell responses to PknB-deficient *S. aureus* cells are not mutually exclusive, and direct as well as indirect mechanisms may be operational simultaneously. In any case, a lack of PknB in *S. aureus* will result in an aberrant regulation of PepG synthesis, which may then lead to reduced bacterial cell lysis, lower amounts of released PepG and/or structural changes in the PepG network. If this is also the case during our infection experiments, both the reduced release of PepG or its structural changes in *pknB* mutant cells might subsequently lead to reduced activation of the PepG-recognizing TLR2, NOD2 and NLRP3 host cell receptors, which are essential for pathogen recognition, downstream kinase signaling and activation of pro-inflammatory processes, such as inflammasome activation and pro-inflammatory cytokine production (Fig. 5)[8,285,286,287]. Importantly, in sepsis not the bacteria *per se*, but the strong inflammatory reaction elicited is the main problem. Therefore, we conclude that our studies on PknB may have provided the first example of a prokaryotic kinase whose inhibition constitutes a rational novel therapeutic avenue in a dangerous infectious disease.

Materials and Methods

Bacterial strains and growth conditions

S. aureus strains NCTC 8325, NCTC 8325 Δ*pknB* and NCTC 8325 Δ*pknB* ΔΦ13 [103] were cultivated overnight (37°C, 250 rpm) in 10 ml of Trypticase Soy Broth (TSB). The overnight cultures were then diluted to an initial optical density at 600 nm (OD_{600}) of 0.08 in 15 ml of TSB and grown at 37°C to an OD_{600} of 0.8, which corresponds to ~ 2.5×10^8 colony forming units (CFU). Bacteria were separated from the growth medium by centrifugation (8000 rpm, 5 min, 4°C), washed three times in phosphate-buffered saline (PBS) and re-suspended in the invasion medium RPMI (Gibco) supplemented with 2 mM L-glutamine (PAA) and 10% heat-inactivated Fetal Bovine Serum (FBS; PAA).

Colony PCR

The sequences for the *sak*, *scn*, *chp* and *hlb* genes of *S. aureus* NCTC 8325 were obtained from the NCBI database (www.ncbi.nlm.nih.gov) in order to design the following primers: *sak* forward, GTTGATATTTATCATCTTAAATAAGG; *sak* reverse, CAAGACATTGCTTTCTATAATAAAC; *scn* forward, CTCATTTTAGGAATTTCGC; *scn* reverse, CAAGTTATGAAATGTCTGCC; *chp* forward, CATTTAATAAGAAGATCTA

TATAGTTAATG; *chp* reverse: CCTATACTGTTTTATTTATGAACAC; *hlb* forward, GTTGTAAGCTATATAAAAGGAGTG; and *hlb* reverse: GAACGAAGCAAGTTAT TAGTTAG. Colony PCR was performed using cells that were grown on tryptic soy agar and blood agar plates. The colonies were touched with a plastic inoculation loop, and resuspended in 200 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). For each reaction 0.5 µl of the colony suspension was used. PCR was performed using a Thermocycler (MJR PTC-200, MJ Research) and amplified fragments were analyzed by agarose gel electrophoresis.

Cell culture and growth conditions

THP-1 cells [191] were grown in suspension in RPMI 1640 medium (Gibco) supplemented with 2 mM glutamine (PAA) and 10% heat-inactivated FBS (PAA). Cell culturing and all subsequent experiments were carried out at 37°C under 5% CO_2. Cells were passaged every three days. Cell viability as determined by the trypan blue exclusion assay [192] was at least 90% before and during all experiments. For phosphorylation profiling assays, 10^5 cells were seeded in 6-well plates (TPP). Differentiation and adherence of THP-1 monocytes was induced by incubation with 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) for 2 days at 37°C in 5% CO_2 [193]. After 2 days of differentiation, the medium was removed, cells were washed three times with PBS, and then the cells were incubated for one more day in fresh supplemented RPMI 1640 medium.

Infection experiments for kinase profiling

THP-1 macrophages were infected with *S. aureus* strains NCTC 8325 Δ*pknB*, *S. aureus* NCTC 8325 Δ*pknB*ΔΦ13 or the parental strain NCTC 8325 at a MOI of 1:50. Macrophages were incubated with bacteria for 15 min, 1 h or 24 h. As a negative control macrophages were incubated without bacteria. After 1 h, medium with non-internalized bacteria was removed, cells were washed three times in PBS, and fresh RPMI 1640 medium supplemented with 2 mM glutamine, 10% heat inactivated FCS and 100 µg/ml gentamycine was added, and macrophages were incubated for another 23 h. After this incubation period, the medium was removed and cells were washed three times in PBS (PAA). The cells were then lysed with Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific), supplemented with Halt Protease and Phosphatase Inhibitor Cocktails (Thermo Scientific), for 1 h on ice under gentle shaking. Lysed cells were scraped from the

plates, collected in fresh Eppendorf tubes and centrifuged for 10 min (14000 rpm, 4°C) in order to remove cellular debris. Then the supernatant was collected and the protein concentration was determined using the Micro BSA™ Protein Assay Kit (Thermo Scientific). All kinase profiling experiments were done in triplicate.

Kinase profiling array analyses

For kinase profiling, we employed the entire complement of kinase substrates described in phosphobase, which were spotted on hydrogel-coated glass as described by Diks *et al.* [116]. This resulted in slides that display 1,152 individual kinase substrates (*i.e.* peptides) in duplicate. The sequences of these peptides and the source proteins from which they were derived can be found in the Supplemental Tables 1 and 2 and, in more detail, on http://www.pepscanpresto.com/files/PepChip%20Kinase%20Map%20File%200103.xls. Procedures for performing phosphorylation profiling and for data analysis were previously described in detail by Diks *et al.* and Miller *et al*. [116,196]. Western blotting to verify kinase profiling results was performed as described by Miller *et al*. [1,116].

Isolation of human peripheral blood mononuclear cells (PBMCs) and stimulation of cytokine production

Isolation of human PBMCs from three healthy donors was performed as previously described with minor modifications [288]. Specifically, PBMCs ($5x10^5$) in a 100µl volume were added to round-bottomed 96-well plates (Greiner) and incubated with either 100 µl of fresh culture medium or various stimuli including *S. aureus* cells (MOIs; 1:10, 1:25 or 1:50), highly purified LPS from *Escherichia coli* at 10 ng/ml [289], or palmitoyl-3-cysteine (Pam_3Cys) 10µg/ml. PBMCs were incubated with stimuli for 1 h, 4h, 8h or 24 h. After addition of either of these stimuli, the plates with PBMCs were centrifuged for 7 minutes at 1,200 rpm. As a negative control, PBMCs that had not been incubated with bacteria were used. After 1 h, medium with non-internalized bacteria was removed, cells were washed three times in PBS, and fresh RPMI 1640 medium supplemented with 2 mM glutamine, 10% heat inactivated FCS and 100 µg/ml gentamycin was added and PBMCs were incubated for another 3h, 7h or 23 hours. At each time point, supernatants were collected and stored at -80°C until assayed for cytokine determination. All PBMC stimulation experiments were done in duplicate with six biological replicates.

Cytokine determination

Human IL-1β, TNFα (R&D Systems), IL-8, IL-6 and IL-10 (Pelikine Compact, Sanquin) levels in cell supernatants were assayed using ELISA kits according to manufacturers' instructions.

Statistical analysis of cytokine production

Differences in cytokine production between PBMCs incubated with different *S. aureus* strains were tested using unpaired t-tests. Data are expressed as the mean ± standard error of the mean (SEM), unless stated otherwise. P values of less than 0.05 (in comparison with the parental *S. aureus* strain) were considered significant. Correlation efficient are presented in Supplementary figure 2.

Acknowledgements

We thank Knut Ohlsen and S. Donat for providing *S. aureus* NCTC 8325 Δ*pknB.* MM, AD, E.R, and JMD were in parts supported by the CEU projects LSHG-CT-2006-037469 and PITN-GA-2008-215524, and the TI Pharma project T4-213.

Chapter 5

A β-lactamase translocation assay for studying protein secretion by *S. aureus* inside mouse macrophages

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Abstract

The Gram-positive bacterium *Staphylococcus aureus* is known as a dangerous human pathogen. In order to become virulent, *S. aureus* produces and secretes a wide range of virulence factors. These virulence factors need to be actively exported from the cells and in some cases they are post-translocationally modified either for folding into a stable conformation or retention at a particular cellular location. Here we have probed the roles of non-essential protein secretion machinery components in the intracellular secretion of a penicillinase by *S. aureus* cells that have been internalized by macrophages. The results show that macrophages with internalized penicillinase-producing *S. aureus* cells lacking the diacyl-glyceryl transferase Lgt, the disulphide bond oxidase DsbA, the mechanosensitive channel MscL or the SecG and SecY2 secretion channel components display highly elevated intracellular levels of penicillinase activity compared to the parental strain. This indicates that the respective mutant strains either have an increased capacity for intracellular penicillinase secretion, a higher propensity to survive internalization or both.

Introduction

The Gram-positive bacterium *Staphylococcus aureus* is a frequent component of the human microbiota as 20-30% of the human population is asymptomatically colonized with *S. aureus* on the skin, upper respiratory tract, or the gastrointestinal tract. Nevertheless, *S. aureus* is also a leading cause of invasive infections. It successfully subverts the human host by secreting an arsenal of virulence factors that can be expressed when needed at different stages of growth.

Specifically the virulence factors of *S. aureus* include: (a) surface proteins that promote adhesion and the colonization of host tissues (e.g. fibrinogen-binding protein, fibronectin-binding protein, collagen-binding protein), (b) invasins that are secreted to promote bacterial spreading in tissues (e.g. leukocidin, hualuronidase), (c) surface factors that inhibit phagocytic engulfment and promote immune evasion (e.g. capsule, protein A, Sbi, coagulase, clotting factor), (d) anti-oxidative compounds and proteins that enhance staphylococcal survival in phagocytes (e.g. carotenoids, catalase), (e) membrane-damaging toxins that destroy eukaryotic cell membranes (e.g. α-, β-, and γ-hemolysin, leukotoxins), (f) superantigens that contribute to the symptoms of septic shock (staphylococcal entertoxin A-G, Toxic shock syndrome toxin TSST, exfoliative toxins A and B), and (g) determinants for resistance to antimicrobial agents (penicillin-binding proteins, penicilinase) [114,115,154,290,291].

Most of the proteinaceous virulence factors of *S. aureus* are displayed on the surface of the staphylococcal cell or released into the extracellular milieu. In general, proteins that have to be secreted from the bacterial cell are synthesized with an N-terminal signal peptide, which directs the newly synthesized protein from the ribosome to the translocation machinery in the cytoplasmic membrane. In *S. aureus* at least six secretion pathways have been identified [221].

In Gram-positive bacteria, such as *S. aureus*, proteins synthesized without a signal peptide generally reside in the cytoplasm, although such proteins can also be encountered bound to the cell surface or in the extracellular environment [292]. This probably relates to cell lysis although active transport mechanisms cannot be excluded. In contrast, proteins with a signal peptide are targeted to the membrane via the Sec, Tat or Com pathways. If the signal peptide remains attached to the translocated protein, this protein will remain attached to the

membrane. However, if the signal peptide is cleaved by signal peptidase during or shortly after membrane translocation, the protein will be released from the membrane. If such a processed protein contains no retention signals for the membrane or cell wall, it will be secreted into the environment. However, if it contains a so-called lipobox, the protein will be lipid-modified and by means of the lipid modification it will remain attached to the membrane. Other translocated and processed proteins contain cell wall retention signals that facilitate their covalent linkage or high-affinity binding to cell wall components [292].

The most commonly used pathway for bacterial protein secretion is the Sec pathway. Proteins that are exported via the Sec pathway contain signal peptides with recognition sites for so-called type I (SpsB) or type II (LspA) signal peptidases (SPases). The process of Sec-dependent protein export can be divided into three stages. Firstly, the proteins are targeted to the membrane translocation machinery by export-specific or general chaperones. Secondly, their translocation across the membrane is facilitated by the Sec machinery, which is composed of the channel components SecY, SecE and SecG, and the translocation ATPase SecA. Notably, the SecY and SecA components are duplicated in *S. aureus* [221,293]. The third and final stage involves processing of the translocated protein by SPase, and post-translocational folding and modification. It should be noted that the Sec pathway can only facilitate membrane passage of proteins in an unfolded state [294,295,296]. While most translocated proteins seem to have an intrinsic capability to fold into their native conformation, their post-translocational folding *in vivo* is usually catalyzed by chaperones and other folding catalysts, such as PrsA and DsbA [221,226,297]. PrsA is a general folding catalyst with peptidyl-prolyl cis/trans isomerase activity that was shown to be involved in the correct folding of several secreted enzymes in *B. subtilis* [298] and the protective antigen of *B. anthracis* [299]. DsbA is a homologue of the *E. coli* DsbA and *B. subtilis* BdbD proteins, which are both oxidases involved in the formation of disulfide bonds in exported proteins [300]. However, unlike its homologues in *E. coli* and *B. subtilis*, the *S. aureus* DsbA does not seem to require a membrane-embedded partner protein for its re-oxidation during catalysis. Instead, this protein is re-oxidized by components in the extracellular environment [301,302]. Bacteria express a wide variety of disulphide-bonded virulence factors, including secreted toxins, surface components, such as adhesins and pili, and secretion systems [303,304]. To be

functionally active, many of these proteins require to be oxidatively folded [305]. Accordingly, the inactivation of genes encoding the Dsb system in cells of various pathogenic microbial species, such as *Vibrio cholerae, Pseudomonas aeruginosa, Salmonella enterica* or *Helicobacter pylori*, reduces their virulence [306,307,308,309].

In addition to the Sec pathway, Gram-positive bacteria can use several other special purpose pathways for protein export from the cytoplasm. These include the Twin-arginine translocation (Tat) pathway, the Com pathway, the YidC pathway, the Ess pathway and secretion via holins or ABC transporters [221,294,296]. The Tat translocase of *S. aureus* consists of the TatA and TatC subunits. This translocase recognizes a twin-arginine motif that consists of two adjacent (twin) Arg residues followed by another amino acid and two hydrophobic amino acids (R-R-x-ϕ-ϕ, where ϕ is a hydrophobic amino acid) [310,311]. In contrast to the Sec translocase, the Tat translocase is able to transport folded proteins across the membrane, which seems of particular relevance for the export of proteins with bound co-factors. The Tat pathway has been studied very well in *E. coli* and *B. subtilis* [312,313,314], but fairly little is known about the roles of this pathway in *S. aureus*. Recently, the Tat pathway of *S. aureus* has been studied using biochemical and proteomics approaches, but this has led to the identification of only one substrate so far, which is FepB [315,316].

The Com pathway of *B. subtilis* is used for the export and assembly of pseudopili that are necessary for DNA binding and uptake during natural genetic competence development [226,297]. Homologues of some of the Com pathway components and pseudopilins are also present in *S. aureus*. So far, limited information is available about this pathway in *S. aureus*. Morikawa and colleagues have shown that the expression of several pseudopilin genes is under control of the SigH sigma factor [317], but whether these genes are involved in DNA uptake has so far remained unclear.

S. aureus and related Gram-positive bacteria have several mechanisms for the retention of proteins that have been exported from the cytoplasm. One of these mechanisms is to bind the protein to the membrane via a lipid anchor. As indicated above, such lipoproteins are synthesized with a Sec-type signal peptide containing the so-called lipobox [318]. The lipobox has the consensus sequence $^{-3}$L-x-x-C^{+1} and contains an invariant Cys residue at the +1 position. The lipobox is

recognized by the diacylglyceryl transferase Lgt and the invariant Cys is then modified by transferring a diacylglyceryl group from phosphatidylglycerol to the sulfhydryl group of this Cys residue [226]. Upon cleavage by the lipoprotein-specific signal peptidase II (LspA), the lipid-modified Cys residue serves as the membrane anchor for the mature lipoprotein. It has been shown that *S. aureus* Lgt is important for virulence and recognition by the host [316]. Furthermore, it was reported that *S. aureus* variants lacking lipoproteins escaped immune recognition and caused lethal infections with disseminated abcess formation in mice. Moreover *lgt*-deficient *S. aureus* failed to provoke a strong host cell activation [72,73,316,319]. However, the molecular basis for these different observations is presently not clear.

Another mechanism employed for the specific retention of proteins in the cell envelope is their covalent attachment to the cell wall. In *S. aureus* and many other Gram-positive pathogens, this reaction is catalyzed by sortases [320,321,322]. Based on phylogenetic analyses of 61 sortases, at least four classes of sortase (A-D) can be distinguished [320]. In the genome of *S. aureus* two genes encoding sortases are present. These enzymes recognize proteins with a C-terminal signal, which consists of the recognition motif (LPxTG), followed by a stretch of hydrophobic residues and several positively charged residues (Arg or Lys). SrtA recognizes proteins with an LPxTG or LPxAG motif and cleaves N-terminally of the Gly residue. At the same time, SrtA couples the mature protein through its new C-terminal Thr or Ala residue to the peptidoglycan in the cell wall [323,324]. In *S. aureus* 21 proteins have been identified with SrtA signals. For several of these proteins (e.g. protein A, ClfA, SasG) it has been shown that they are indeed attached to the cell wall by SrtA, and that they are involved in virulence [325,326]. The gene encoding the second sortase of *S. aureus*, SrtB, lies in an operon that also encodes the SrtB substrate IsdC and several other genes involved in iron acquisition [327]. The structure of this operon is conserved in other Gram-positive pathogens, such as *Bacillus anthracis*, *Bacillus cereus* and *Listeria monocytogenes* [320]. IsdC contains a C-terminal NPQTN SrtB recognition motif instead of the SrtA recognition motifs LPxTG or LPxAG [328]. The different substrate specificities of SrtA and SrtB relate to differences in their substrate binding pockets [329]. Staphylococcal SrtA is highly resistant to oxidative inhibition, both *in vitro* and *in vivo*. Therefore, SrtA can resist the extreme

conditions encountered in the phagosome, which may contribute to intracellular survival of *S. aureus* inside phagocytes [330].

For many years *S. aureus* was considered to be an extracellular pathogen. However, recent reports have shown that this organism can also survive intracellularly [62,65,66]. For example, it was reported that *S. aureus* can survive in endothelial and epithelial cells [59] as well as in osteoblasts [60] thereby causing persistent infections, such as endocarditis [61], and osteomyelitis. Furthermore *S. aureus* redirects from the endosomal pathway to autophagosomes. In this way *S. aureus*-induced autophagy is required for staphylococcal replication, subsequent escape from autophagosomes into the cytoplasm, and eventually *S. aureus*-induced host cell death [64]. Moreover, it is now known that professional phagocytes provide a beneficial environment for intracellular survival of *S. aureus*. Consistent with this notion, recent studies have revealed that *S. aureus* is highly resistant to neutrophil- and macrophage-mediated killing [65,66].

Protein secretion by *S. aureus* has been studied in substantial detail through experiments involving *in vitro* cultivation, and the effects of mutations in secretion machinery components on staphylococcal virulence have been assessed in animal experiments. However, the actual protein secretion process has thus far not been directly monitored *in vivo* due to the lack of appropriate assays. One way of approaching this challenge is to study protein secretion by *S. aureus* cells upon their internalization by phagocytic cells, which was the objective of the present studies. For this purpose, we used a β-lactamase activity assay that allows the detection of staphylococcal penicillinase secretion inside mouse macrophages. Using this assay, we assessed the intracellular penicillinase secretion potential of a collection of *S. aureus* mutant strains lacking various secretion machinery components.

Results and Discussions

To monitor penicillinase secretion by *S. aureus* cells that have been internalized by mouse macrophages, we made use of the coumarin cephalosporin fluorescein dye (CCF2-AM). Upon cleavage of the β-lactam ring of CCF2-AM by penicillinase, this dye no longer exhibits fluorescence resonance energy transfer (FRET), resulting in a shift in the emission spectrum from green to blue. Infected macrophages can be loaded with CCF2-AM and cleavage of this dye can then be monitored through

fluorescence microscopy [331,332]. Accordingly, blue macrophages would be indicative for the secretion of active penicillinase by internalized cells of *S. aureus*. Indeed, when J774 mouse macrophages were incubated for 24 h with the penicillinase-producing MRSA strain E91 at a multiplicity of infection (MOI) of 1:50, we observed a significant number of blue cells upon loading with CCF2-AM. In contrast, CCF2-AM-loaded macrophages that had not been incubated with *S. aureus* remained green under the tested conditions (Fig. 1, top panels). This showed that the CCF2-AM assay for the detection of β-lactamase activity also works for penicillinase secretion studies with *S. aureus*.

To investigate the role of different secretion machinery components in intracellular penicillinase secretion, *S. aureus* strain SH1000 and 13 mutant derivatives of this strain lacking individual genes for particular secretion machinery components were transduced with plasmid pE91 from strain E91 as this plamid carries a highly expressed gene for a secreted penicillinase. Next, we incubated J774 mouse macrophages with the different pE91-containing *S. aureus* strains at a multiplicity of infection (MOI) of 1:50. Upon 24 h incubation, the macrophages were loaded with CCF2-AM and the numbers of blue cells were determined. As anticipated, penicillinase secretion by the internalized parental strain *S. aureus* SH1000 carrying pE91 resulted in the appearance of blue macrophages, whereas no blue macrophages were observed upon incubation with the *S. aureus* SH1000 strain that lacked pE91 (Fig. 1). However, the number of blue macrophages observed upon incubation with the SH1000 strain carrying pE91 was relatively low (~2%) compared to the number of blue macrophages observed upon incubation with the penicillinase-producing E91 strain. The reason for this difference is presently not clear, but it may relate to differences in the levels of penicillinase production. However, since we were unable to delete the genes for secretion machinery components from the E91 strain for technical reasons, we used secretion mutant derivatives of strain SH1000 for all further analyses.

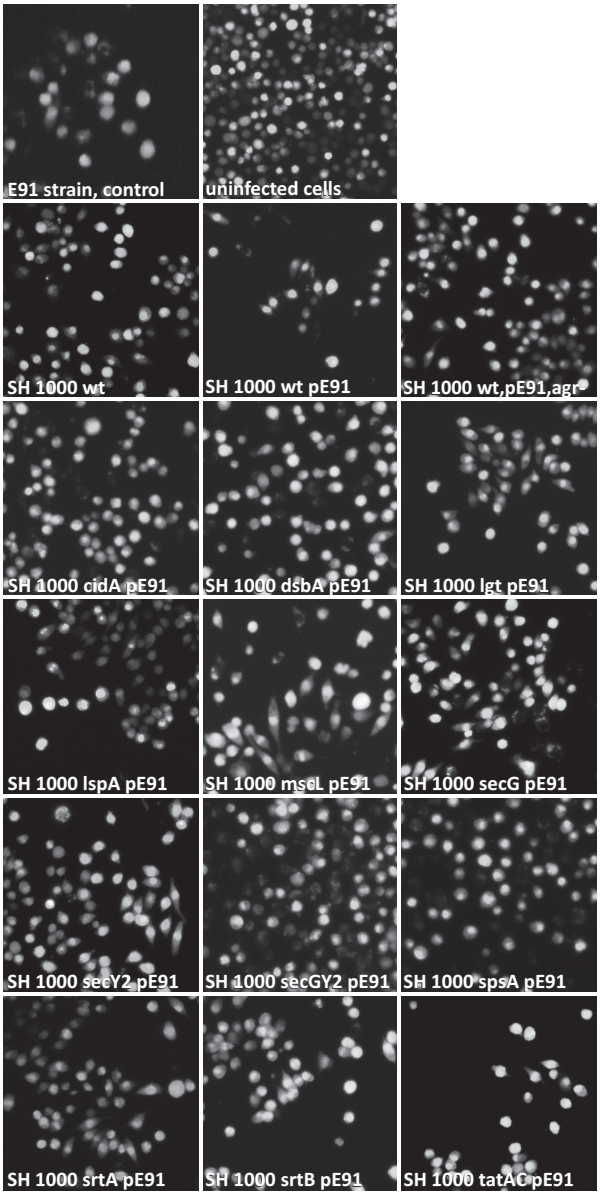


Figure 1. Secretion of penicillinase into J774 macrophages by different strains of *S. aureus*. The secretion of an active penicillinase by wild-type *S. aureus* or 13 mutant *S. aureus* strains with different protein secretion defects was assessed by fluorescence microscopy. For this purpose, the infected macrophages were loaded with CCF2-AM. Cells in which intracellular secretion of penicillinase has occurred appear blue.

In previous studies we had observed that spontaneous *agr* mutant derivatives emerge from the SH1000 strain at a relatively high frequency [333]. To rule out any effects of potentially emerging *agr* mutants, we also tested the penicillinase secretion assay with an *agr* mutant derivative of strain SH1000 carrying pE91, but there was no significant difference with the *agr* proficient strain detectable (Figs. 1 and 2). Next, we tested intramacrophage penicillinase secretion by mutants lacking particular genes implicated in protein secretion. Compared to the parental strain SH1000, the strains lacking the *cidA*, *lspA*, *secG*, *secY2*, *spsA*, *srtA*, *srtB* or *tatAC* genes showed no significant difference in the generation of blue macrophages, suggesting that, upon internalization, they secreted penicillinase to the same extent as the parental strain. This finding also implies that there were no major differences in the intracellular survival of these mutant strains under the tested conditions. However when mouse macrophages were incubated with Δ*lgt*, Δ*dsbA*, Δ*mscL* or Δ*secG*Δ*secY2* mutant *S. aureus*, we observed significantly more blue macrophages (~8%, ~10%, ~6% and ~35% respectively) than when macrophages were incubated with the parental strain (Figs 1 and 2). The staphylococcal diacyl-glyceryl transferase Lgt recognizes the lipobox of lipoprotein precursors and catalyzes the diacyl-glyceryl modification of the Cys residue in the lipobox. This enzyme is therefore required for binding of mature lipoproteins to the membrane. Furthermore, the non-modified lipoproteins of bacteria that lack Lgt are often poorly retained in the cell envelope, which leads to their accumulation in the growth medium (Tjalsma et al., 2004, MMBR). Therefore the observed increase in the secretion of penicillinase by the Δ*lgt* mutant suggested that the investigated staphylococcal penicillinase specified by pE91 might be a lipoprotein. To answer this question, we sequenced pE91, which revealed that the encoded penicillinase is indeed a lipoprotein with the lipobox core sequence $^{-3}$L-S-A-C^{+1} and conserved adjacent residues (Fig. 3). By analogy to previous observations on *lgt* mutant cells grown in broth [73,334], we conclude that the enhanced frequency of blue macrophages upon infection with the Δ*lgt* mutant *S. aureus* cells is due to the enhanced release of penicillinase into CCF2-AM-loaded macrophages. Notably, Lgt was also shown to be important for the recognition of *S. aureus* by the host and for virulence [72,73,316,319].

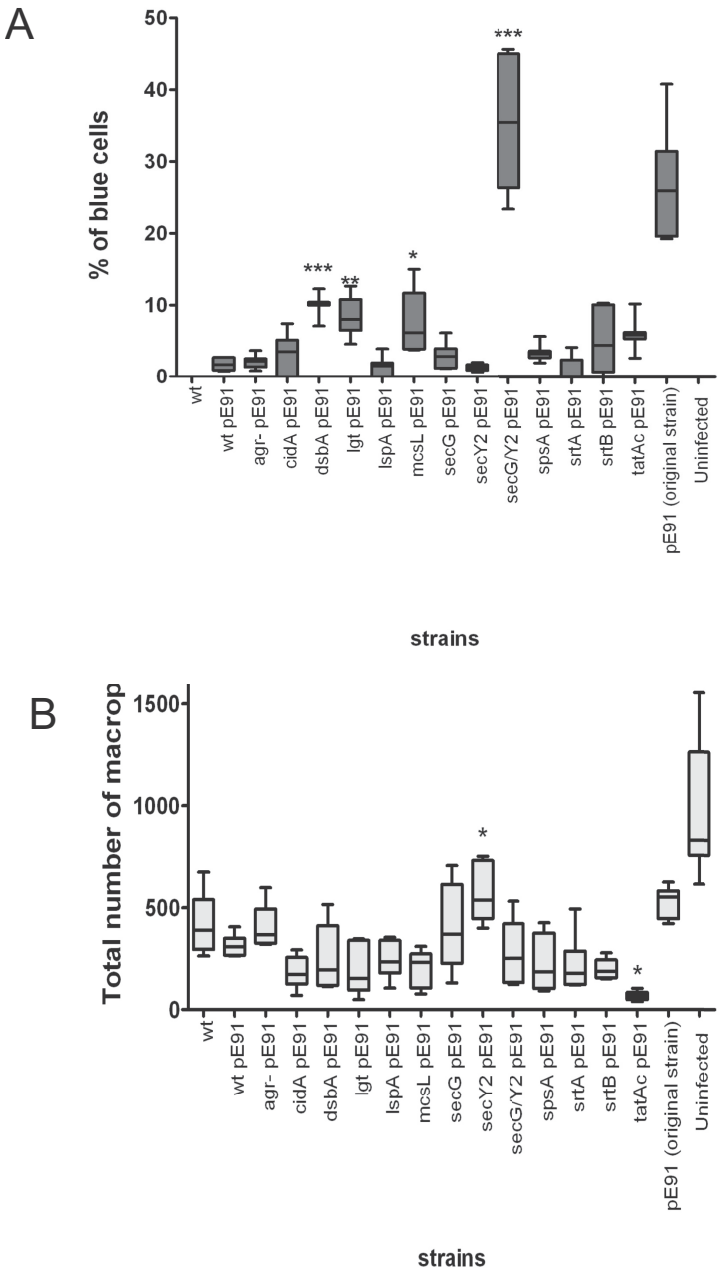


Figure 2. Quantification of penicllinase translocation into J774 macrophages by different strains of *S. aureus*. Cells from six independent random microscope fields were counted. The percentage of blue cells (A) and the total number of macrophages was calculated (B). The results shown represent the mean ± SD of six microscope fields.

In these studies, *lgt*-deficient *S. aureus* cells did not induce host cell activation, but escaped immune recognition and caused lethal infections in mice. Interestingly, these observations could also explain the increased number of blue macrophages that were observed upon infection with the Δ*lgt* mutant cells. In this case, the bacterial cells lacking lipid-modified lipoproteins would not be properly recognized by the host receptors and would therefore not trigger host inflammatory responses. Accordingly, the killing of internalized *lgt*-deficient cells might be less effective than the killing of *lgt*-proficient cells. This would result in increased intracellular survival of Δ*lgt* mutant *S. aureus* cells and, accordingly, the amounts of released active non-modified penicillinase would be increased. At present we cannot distinguish between these two possibilities.

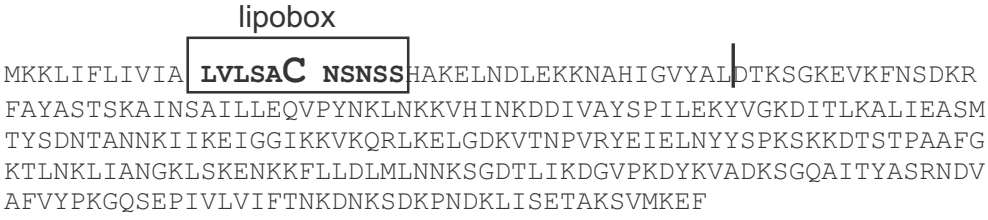


Figure 3. Amino acid sequence of the penicillinase encoded by plasmid pE91. Sequencing of plasmid pE91 revelead that the pE91-encoded penicillinase is a lipoprotein with the lipobox core sequence -3L-S-A-C+1 and conserved adjacent residues.

Increased numbers of blue macrophages were also observed when macrophages were incubated with *dsbA*-deficient *S. aureus* (Fig. 1 and 2A). DsbA is a lipoprotein that can catalyze disulphide bond formation in newly synthetized and exported proteins, although substrate proteins for disulphide bond formation have not yet been identified in *S. aureus* [335,336]. Inactivation of the *S. aureus dsbA* gene might however result in the secretion of non-active virulence factors or some virulence factors might be less stable due to the lack of disulphide bonds resulting in increased sensitivity to proteases. Notably, we did not observe increased survival of macrophages with internalized *dsbA* mutant *S. aureus* cells (Fig. 2B), so if a virulence factor were to be affected by the *dsbA* mutation, it would have to have a role in the survival of internalized *S. aureus* cells. In turn, this would result in higher numbers of blue macrophages. Consistent with this idea, it has previously been reported that disulphide bonding plays a critical role

in the virulence of Gram-negative bacteria [337,338]. Notably, it is also conceivable that DsbA catalyzes disulphide bond formation between the cysteine residues in the lipoboxes of two penicillinase precursor molecules prior to their lipid modification by Lgt. If so, this disulfide bond would preclude the lipid-modification of the Cys residues in the two lipoboxes as well as separation of the signal peptides and the 'mature proteins' by the lipoprotein-specific SPase Lsp. In this case, deletion of the *dsbA* gene would result in lipid modification of the non-disulphide bonded penicillinase precursors and subsequent processing by Lsp. However, this would not directly explain the increased levels of blue macrophages as observed upon internalization of *dsbA* mutant cells. Therefore, we consider it more likely that internalized *dsbA* mutant cells are less well recognized and killed by the macrophages.

The internalization of *mscL*-deficient *S. aureus* cells also yielded increased numbers of blue macrophages (~6%). The *mscL* gene encodes the 'large mechano-sensitive channel' that opens when the cells build up a high turgor pressure, which is the case upon hypo-osmotic shock. It has been reported that under hypo-osmotic shock conditions, normally cytoplasmic proteins were specifically released by *mscL*-deficient cells in *Bacillus subtilis*. Thus it seems that MscL prevents the release of particular cytoplamic proteins by *B. subtilis* [339]. Moreover MscL plays a critical role in the susceptibility of *S. aureus* to the glycopeptide sublancin 168 especially at low-salt concentrations. Specifically, it was shown that *S. aureus* was able to survive only for a few hours in the presence of the sublancin 168-producing *B. subtilis* strain 168 in LB medium containing 1% NaCl. In contrast, co-cultivation of *S. aureus* with *B. subtilis* 168 in LB with 5% of NaCl did not have any effect on staphylococcal growth. Importantly, co-culturing of *S. aureus* lacking *mscL* with *B. subtilis* producing sublancin 168 in LB with 1% NaCl did not reveal any growth inhibition of the *S. aureus* Δ*mscL* strain. These observations demonstrate that the susceptibility of *S. aureus* to sublancin 168 is dependent on the presence of an MscL channel [340]. These findings, together with the fact that MscL has not been implicated in lipoprotein release, suggest that *S. aureus* cells lacking MscL are better able to survive the internalization in mouse macrophages than MscL-proficient *S. aurues* cells. For example, *mscL* mutant cells might be more resistant to host defensins, analogous to their resistance to sublancin 168. In this case, the hypo-osmotic pressure that is formed in phagocytes might open the MscL channel, allowing phagosomal antimicrobial

peptides to enter the bacterial cells and to kill them. *S. aureus* cells lacking MscL channels would then be less susceptible to antimicrobial peptides, which would increase their survival [341,342].

Our results have also revealed increased numbers (~35%) of blue mouse macrophages upon incubation with the *S. aureus* Δ*secG*Δ*secY2* double mutant. Interestingly, the percentage of blue cells incubated with the positive control strain *S. aureus* E91 was substantially lower (~25%; Figs. 1 and 2). SecG and SecY2 are components of the Sec machinery [343,344]. SecA binds and hydrolyzes ATP, and pushes the preproteins through the SecYEG channel. It has been shown for *S. aureus*, that both SecA and SecE and SecY are essential for growth and viability [345]. In addition, *S aureus* has also a second set of *secA* and *secY* genes in its genome, referred to as *secA2* and *secY2.* Recent studies of Sibbald *et. al* have shown that *secG* and *secY2* are not essential for the growth and viability of *S. aureus* when cultivated in broth. Nevertheless, the absence of *secG* caused a serious decrease in the amounts of the cell wall-bound Sbi protein. In contrast, *secG secY2* double mutant cells secreted increased amounts of certain cell wall proteins, namely staphylococcal protein A (Spa), the peptidoglycan hydrolase LytM, the immunodominant staphylococcal antigen (Isa), and staphylococcal secretory antigen A (SsaA). Conversely, reduced amounts of proteins without retention signals were secreted by the Δ*secG*Δ*secY2* double mutant [293]. Staphylococcal protein A (SpA), is a protein that is covalently anchored to the cell wall of *S. aureus* and that has the ability to interact with human IgG in a non-antigen specific manner [346]. Recent studies have shown that the expression of SpA on the bacterial cell surface makes the bacterium less susceptible to phagocytosis by human neutrophils in the presence of human serum, possibly as a result of the IgG Fc binding property of SpA [347,348]. Moreover, SpA was shown to contribute to a more severe disease outcome with respect to both arthritis and septic death in *S. aureus*-infected mice [42]. While peptidoglycan hydrolases like LytM play important roles in growth, turnover, and cell lysis, a direct role of LytM in *S. aureus* virulence is still unknown [349]. The results of Sibbald *et. al* have also revealed increased amounts of IsaA and SsaA in the medium of Δ*secG*Δ*secY2* deficient *S. aureus* strains, but also for these proteins the function in staphylococcal virulence is not clear and has yet to be determined. Nevertheless Isa and SsaA have been reported to play a role in eliciting an immune responses during sepsis and endocarditis [350,351]. Possibly, the balance between increased

secretion of SpA, LytM, IsaA and SsaA and reduced secretion of other proteins is favorable for survival inside macrophages, which would explain why we observed higher numbers of blue macrophages upon infection with *secG secY2* double mutant cells.

In conclusion, the present studies have addressed the roles of different protein secretion pathways and their individual components in the intra-macrophage survival of *S. aureus* and the secretion of active penicillinase by this organism. Increased numbers of macrophages displaying intracellular penicillinase activity were observed upon incubation with *S. aureus* cells lacking Lgt, DsbA, MscL or the Sec pathway components SecG and SecY2. While these phenotypes are very clear, it is presently not possible to distinguish unambiguously between effects that are caused by increased intracellular penicillinase secretion and effects on the intracellular persistence of *S. aureus*. To make this distinction, future studies will address the effects of *lgt*, *dsbA*, *mscL* and *secG*/*secY2* mutations on the intramacrophage persistence of *S. aureus*.

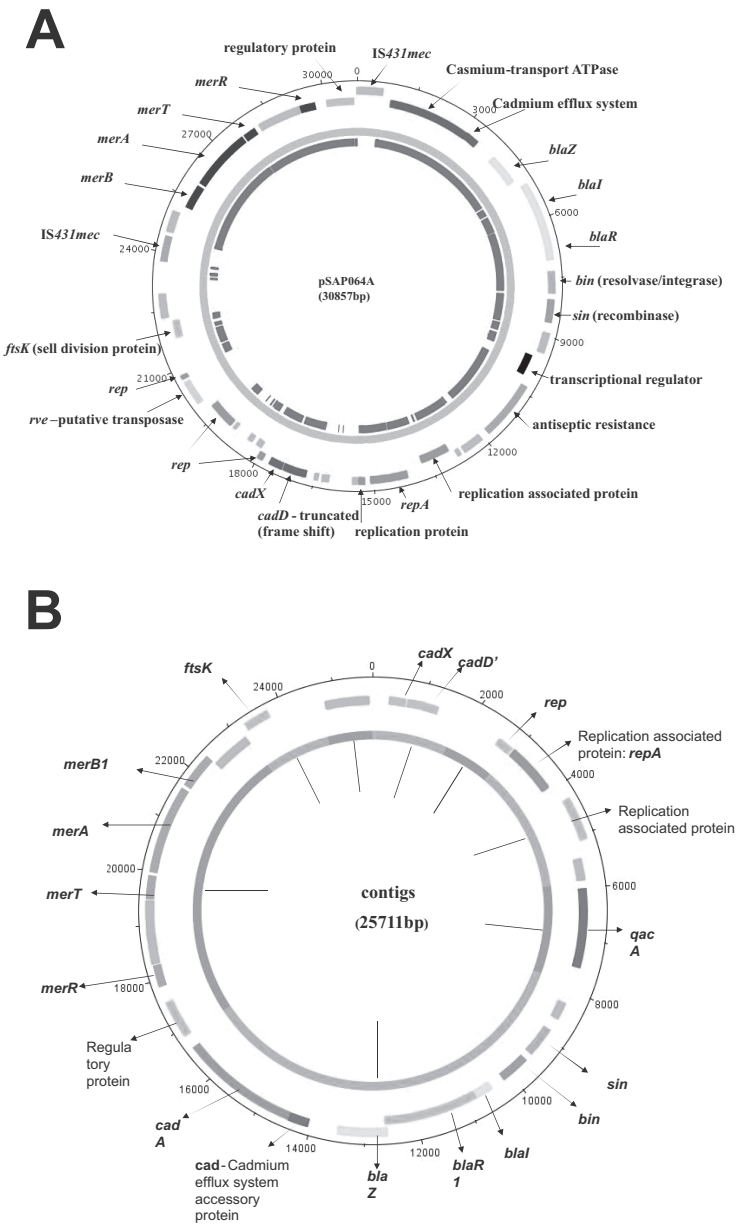


Figure 4. **(A)** Schematic circular diagram of the reference plasmid pSAP064A (outer circular diagram). Known CDSs are annotated and colored. Hypothetical proteins are represented by light blue blocks. The inner concentric red circle represents FASTA best matches shared between the reference plasmid and data obtained from plasmid sequencing of strain E91. **(B)** The hypothetical structure of the sequenced plasmid from strain E91 is shown by a schematic circular diagram. The outer circular diagram represents annotated CDSs also marked with different colors. Open reading frames of unknown function are indicated by light blue. The inner circular diagram represents ordered contigs obtained from the assembly.

Materials and Methods

Bacterial strains, phages and plasmids.

All strains used in this study are listed in Table 1. *S. aureus* strains were grown at 37°C in tryptic soy broth (TSB), under vigorous shaking, or on trypic soy agar (TSA) plates or B plates. The phage ϕ53 and its propagating *S. aureus* strain (PS strain) were obtained from Prof. V. Hajek (Palacky University, Olomouc, Czech Republic). *S. aureus* ISP8 was obtained from Prof. P. A. Pattee (Iowa State University, Ames). The phage ϕ53 was propagated as described by Blair *et al.* [352]. The strain ISP8 (pE91) was generated by transduction of strain ISP8 with the penicillinase plasmid pE91 using a ϕ53 lysate derived from the MRSA strain E91 (pE91) (Prof. J. Doskar, Masaryk University, Brno, Czech Republic). The mutated variants of strain SH1000 carrying the penicillinase plasmid pE91 were obtained through transduction with ϕ53 and strain ISP8 (pE91) as the plasmid donor. Transduction experiments were performed at a multiplicity of infection between 0.1 and 1.0 essentially as described by Lindsay *et al*. [353]. Selective plating media were made by adding 1.5% agar to broth along with cadmium nitrate to a final concentration of $2.5\ 10^{-4}$ M.

Construction of *S. aureus* mutant strains.

Mutants of *S. aureus* were constructed using the temperature-sensitive plasmid pMAD [354] and previously described procedures [340,355].

Invasion and penicilinase translocation assay

For invasion experiments, the *S. aureus* strains listed in Table 1 were grown overnight at 37 °C in 10 mL of trypticase soy broth (TSB) under vigorous shaking. The overnight cultures were then diluted to an initial optical density at 600 nm (OD_{600}) of 0.08 in 15 mL of TSB and grown at 37°C to an OD_{600} of 0.8, which corresponds to ~2.5×10^8 colony forming units (CFU). Bacteria were washed three times in TSB and resuspended in TSB. Next, the bacteria were added to previously seeded $1x10^5$ J774.A1 mouse macrophages in 24-well plates at a multiplicity of infection (MOI) of 1:50 (37°C, 5% CO_2). After 1 h, DMEM medium with non-internalized bacteria was removed, the macrophages were washed three times in PBS, and fresh DMEM medium supplemented with 2 mM glutamine, 10% heat inactivated FCS and 100 µg/mL gentamicin was added. Macrophages with internalized bacteria were incubated for another 23 h. After 24 hours in total,

the macrophages were washed once with Hank's balanced salt solution (Invitrogen) and loaded with a solution sontaining the fluorescent substrate CCF2-AM [332] at a final concentration of 1mM for 1,5 h at room temperature using the standard loading protocol recommended by the manufacturer (Invitrogen). Fluorescent microscopic analyses were performed using an Axiovert M200 microscope (Carl Zeiss, Germany) equipped with a CCF2 filter set (Chroma Technology, Brattleboro, VT, USA). Fluorescence micrographs were captured using a Zeiss Axiocam MRC5 and Zeiss AxioVision 4.5 software, as previously described by de Jong *et al* [356].

Sequencing of plasmid pE91

Plasmid pE91, which directs high-level secretion of a staphylococcal penicillinase, was derived from the clinical MRSA strain E91 isolated in Czech Republic. Plasmid DNA was isolated using the Invitek plasmid isolation kit after a 30-min prelysis step with lysostaphin (5 U/µl) (AMBI PRODUCTS) in kit solution A. The purified plasmid DNA was then sequenced with a Hiseq 2000 instrument (Illumina) at the Genome Analysis Facility of the Department of Genetics at the University Medical Centre Groningen. The obtained sequences were analyzed using the CLC-workbench software (CLC-bio) and assembled into contigs. These were compared to nucleoted sequences in the database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez) using the BLAST tool. Gaps between the contigs were not closed.

Table 1. Strains used in the assay for intra-macrophage penicillinase secretion.

E91	*mecA*, *CadAD*, *blaZ,* methicillin resistant, Cd resistant, penicillinase production, *spa* type t051, MLST type ST247	Clinical MRSA strain, This work
ISP8 (pE91)	*CadAD*, *blaZ,* methicillin resistant, Cd resistant, penicillinase production, donor strain for transduction experiments	[355]
SH1000 pE91	*rsbU+ derivative of 8325-4 (rsbU+, agr+), CadAD*, penicillinase production	[357], this work
SH1000 ΔcidA::kan pE91	*cidA, KanR, CadAD*, penicillinase production	[355], this work
SH1000 ΔdsbA pE91	*dsbA, CadAD*, penicillinase production	[355], this work
SH1000 Δlgt pE91	*lgt, CadAD*, penicillinase production	[355], this work
SH1000 ΔlrgA::kan pE91	*lrgA, KmR, CadAD*, penicillinase production	[355], this work
SH1000 ΔlspA pE91	*lspA, CadAD*, penicillinase production	[355], this work
SH1000 ΔmscL pE91	*mscL, CadAD*, penicillinase production	[340,355], this work
SH1000 ΔprsA pE91	*prsA, CadAD*, penicillinase production	[355], this work
SH1000 ΔsecG pE91	*secG, CadAD*, penicillinase production	[293,355], this work
SH1000 ΔsecY2 pE91	*secY2, CadAD*, penicillinase production	[293,355], this work
SH1000 ΔsecGY2 pE91	*secGY2, CadAD*, penicillinase production	[293,355], this work
SH1000 ΔspsA pE91	*spsA, CadAD*, penicillinase production	[355], this work
SH1000 ΔsrtA pE91	*srtA, CadAD*, penicillinase production	[355], this work
SH1000 ΔsrtBpE91	*srtB, CadAD*, penicillinase production	[355], this work
SH1000 ΔtatAC pE91	*tatA tatC, CadAD*, penicillinase production	[355], this work

Chapter 6

Summary and general discussion

Staphylococcus aureus is one of the most common and versatile human pathogens, responsible for a wide range of infectious diseases that can vary in severity from very mild to deadly. Although 30% of the human population is colonized with *S. aureus* on the skin, upper respiratory tract and gastrointestinal tract without any symptoms, in some individuals this bacterium does cause serious invasive infections. *S. aureus* can be such a successful pathogen, because it secretes an arsenal of virulence factors that are expressed when needed during different stages of growth and under different conditions. These include surface proteins assisting in host colonization, invasins and proteases that promote bacterial spreading, surface factors such as the protein A that inhibits phagocytic engulfment, and toxins that damage host cell membranes. Notably, recent reports have focused attention on a eukaryotic-like serine/threonine kinase of *S. aureus* named PknB, which is involved in bacterial metabolism and cell wall synthesis. However, the role that PknB plays in staphylococcal pathogenesis had not been clarified when the studies described in this thesis were initiated. Since eukaryotic-like serine/threonine kinases have been implicated in the virulence of various other bacterial pathogens, it was decided to investigate the role of PknB in the interactions between *S. aureus* and host immune cells.

Pathogenic micro-organisms are first recognized by cells of the immune system through pattern-recognition receptors (PRRs), such as the Toll-like receptors (TLRs) or the NOD receptors. Most bacterial structures are recognized by more than one receptor and these receptors can also interact with each other. Therefore, the activation of various receptors leads to the activation of a complex network of signaling pathways due to cross-talk between the activated receptors and various downstream signaling molecules. In **Chapter 2** the complex interactions between *S. aureus* and human macrophages were deciphered, and a general overview of interactions between these host cells and *S. aureus* proteins is presented. Using kinase profiling, it was shown that the responses of macrophages to *S. aureus* are mediated by a number of receptors, including the G-protein coupled receptor, receptor tyrosine kinases, the scavenger receptor 36 and integrins. At the same time, the results challenge the classical concept that the inflammatory response is activated mainly through Toll-like receptor 2 and NF-κB signaling. Moreover, the results also show that stress-activated MAP kinases, JNK in particular, play important roles in orchestrating the host defence during *S. aureus* infection. To investigate possible interactions between *S. aureus*

and the human macrophages a proteomics approach was employed. By means of gel-free proteomic analyses on non-phagocytosed *S. aureus*, a number *S. aureus*-bound macrophage heat shock proteins, antimicrobial histones, enolase and other proteins were identified. Some of these proteins are components of extracellular traps, which are formed by phagocytes after stimulation with mitogens, cytokines or pathogens. The proteomic analyses also revealed that after 1h of incubation with human THP-1 macrophages, *S. aureus* mounts a "stringent response" due to nutritional stress. The "stringent response" is characterized by a strong decrease of proteins involved in translation and a concomitant increase of proteins responsible for cell metabolism. The results also revealed activation of the σ^B-dependent general stress response as reflected by elevated levels of the RsbV and RsbW proteins and subunits of the ATP-dependent Clp protease. Interestingly the proteomics data also show that *S. aureus* cells grown in the presence of THP-1 macrophages produce the staphylococcal serine/threonine kinase PknB, whereas this protein was not detectable in bacterial cells incubated without THP-1 cells.

While it has been previously shown that staphylococcal Pknb is involved in *S. aureus* cell wall metabolism and antibiotic resistance, no studies had been done to investigate the properties of staphylococcal PknB with respect to its ability to phosphorylate human proteins. Therefore the same peptide microarrays that were used in Chapter 2 were also used to define the specificity of purified PknB. This was a relevant approach, because full-size PknB was detectable in the growth medium of *S. aureus* cells, suggesting that PknB might be able to interact with targets in host immune cells especially upon internalization of *S. aureus* through phagocytosis. The results of this analysis are documented in **Chapter 3**, where purified PknB was used for kinase profiling instead of macrophage lysates. The results show that PknB can indeed recognize and phosphorylate known substrates of human serine/threonine kinases. In addition, it is shown that proline is a part of the PknB recognition and target sequence. This links PknB to the family of proline-directed kinases, which includes cyclin-dependent protein kinases, glycogen synthase kinase -3 and mitogen-activated protein kinases (MAP kinases). The studies described in Chapter 3 thus suggest that active PknB released from invasive *S. aureus* cells may target signal transduction mechanisms for host cell subversion. Therefore, the role of *S. aureus* PknB in the infection of human peripheral blood mononuclear cells (PBMCs) and THP-1 macrophages was studied using enzyme-linked immune sorbent assays (ELISA) and kinase profiling.

The results presented in **Chapter 4** show that infection of human PBMCs with *S. aureus* lacking *pknB* results in reduced secretion of inflammatory mediators, such as interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), and tumor necrosis factor α (TNF-α). To further investigate whether reduced cytokine production can be correlated with reduced activity of host kinases, the kinase profiling assay was employed. The results of kinase profiling show a reduced activity of important pro-inflammatory kinases, such as the Bruton's tyrosine kinase, Focal adhesion kinase and Src-family kinases. Additionally, the results presented in Chapter 4 demonstrate that the staphylococcal phage 13 limits the intensity of macrophage signal transduction responses to *pknB* mutant *S. aureus.* Notably, this phage carries the genes for CHIPS (Chemotaxis Inhibiting Protein of *S. aureus*), SCIN (Staphylococcal complement inhibitor) and staphylokinase. In Chapter 4, three possible scenarios are presented to explain the observed reduced amounts of inflammatory mediators and the downregulation of tyrosine kinases in PBMCs and macrophages incubated with *S. aureus* lacking *pknB*. In the first place, secreted PknB from internalized *S. aureus* cells might recognize and phosphorylate certain host proteins (see Chapter 3), thereby directly impacting on the tyrosine kinase signaling. However, the results described in Chapter 3 do not indicate that PknB can recognize any of the tyrosine kinases targets. This would suggest that secreted PknB can only indirectly impact on tyrosine kinase signalling. Notably, PknB was shown to be a proline-directed serine/threonine kinase with similar activity as the eukaryotic MAP kinases (Chapter 3). Furthermore, the results showed that PknB can recognize and phosphorylate the Activating transcription factor-2, the target of MAP kinases (Chapter 3). However verifying *in vivo* whether this actually happens upon internalization of *S. aureus* will be very difficult, especially since ATF-2 is massively phosphorylated by MAP kinases. It will therefore remain a major challenge to distinguish between the phosphorylation mediated by MAP kinases and PknB. The same applies for the phosphorylation of other proteins, which were shown in kinase profiling to be recognized by the staphylococcal PknB. A second explanation for the reduced inflammatory responses towards *S. aureus pknB* mutant cells, is the reduced activity of PknB-regulated autolysins and reduced cell turnover of *S. aureus* lacking an intact *pknB* gene. Therefore, one can speculate that reduced cell wall turnover in the *pknB* mutant bacteria results in the release of lower amounts of peptidoglycan from the bacterial cells. In turn, this might

affect the recognition of bacterial peptidoglycan or whole bacteria by the THP-1 macrophages and PBMCs. A third reason for the reduced responses of human cells to *pknB* mutant *S. aureus* might be the possible structural change(s) in peptidoglycan due to the absence of PknB, which could affect certain peptidoglycan-lipoteichoic acid interactions. These interactions are responsible for the synergism and proper *S. aureus* recognition that leads to the onset of inflammatory responses. To conclude, the present data clearly show that *S. aureus* requires PknB to provoke strong inflammatory responses. Therefore, specific PknB inhibitors that are yet to be developed may find future applications in combating the fulminant pathology of *S. aureus* infections.

A major question that remained to be answered concerned the role of PknB in the intracellular survival of *S. aureus* upon phagocytosis. In **Chapter 5** the secretion of a penicillinase by *S. aureus* cells was investigated upon their internalization by mouse macrophages. For this purpose, a β-lactamase activity assay was employed that allows the detection of staphylococcal penicillinase secretion inside macrophages by fluorescence microscopy. Using this assay, the role of different secretion machinery components and PknB was investigated in intracellular penicillinase secretion and *S. aureus* survival (Fig. 1 and Fig. 2; not shown in Chapter 5 and). The results show that mutant cells lacking the *lgt*, *dsbA*, *mscL* or *secG* and *secY2* genes yielded more macrophages with penicillinase activity than the parental strain. This suggests that the absence of the diacyl-glyceryl transferase Lgt, the thiol-disulphide oxidoreductase DsbA, the mechanosensitive channel MscL, or the SecG and SecY2 translocation channel components results in improved intracellular survival of the mutant cells, in elevated levels of penicillinase release into the macrophages, or both. Importantly, the absence of PknB did not detectably influence the penicillinase activity observed in macrophages (Fig. 1), which implies that the survival of *pknB* mutant *S. aureus* cells inside mouse macrophages was not affected. Taken together, the present studies provide first insights into the complex network of signaling reactions that unfold when pathogenic *S. aureus* and host immune cells collide.

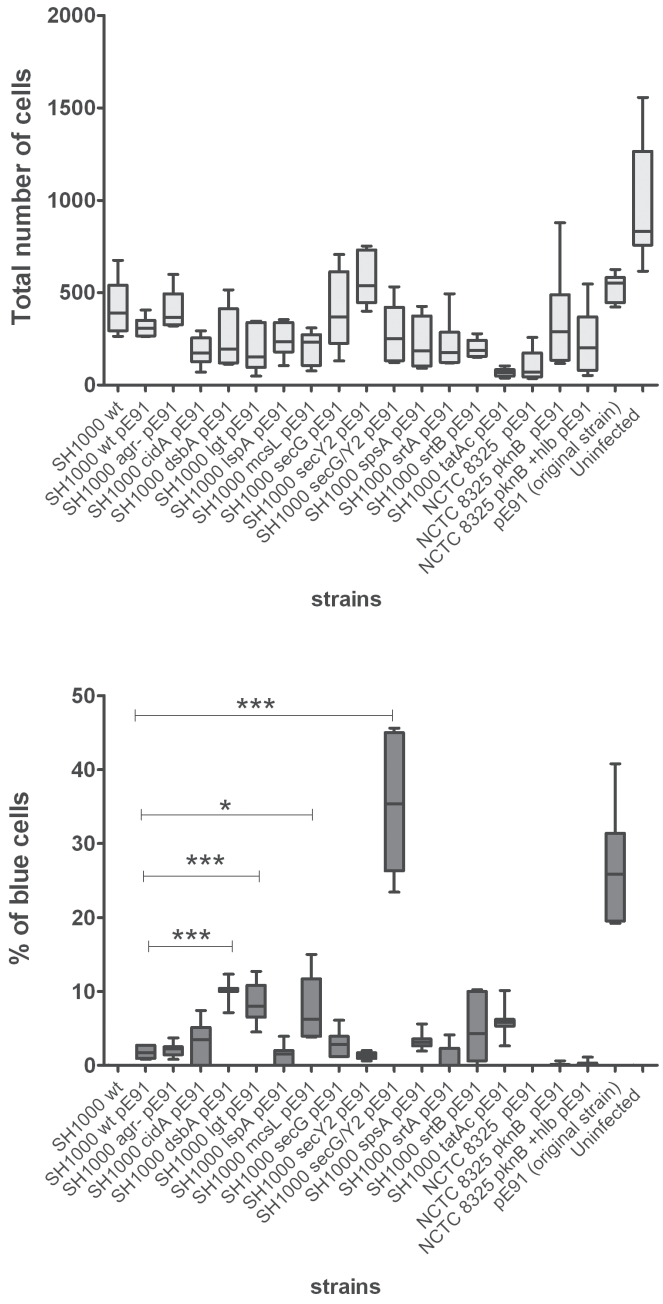


Figure 1. Quantification of penicillinase translocation into J774 macrophages by different strains of *S. aureus*. Cells from six independent random microscope fields were counted. The percentage of blue cells (A) and the total number of macrophages was calculated (B). The results shown represent the mean ± SD of six microscope fields.

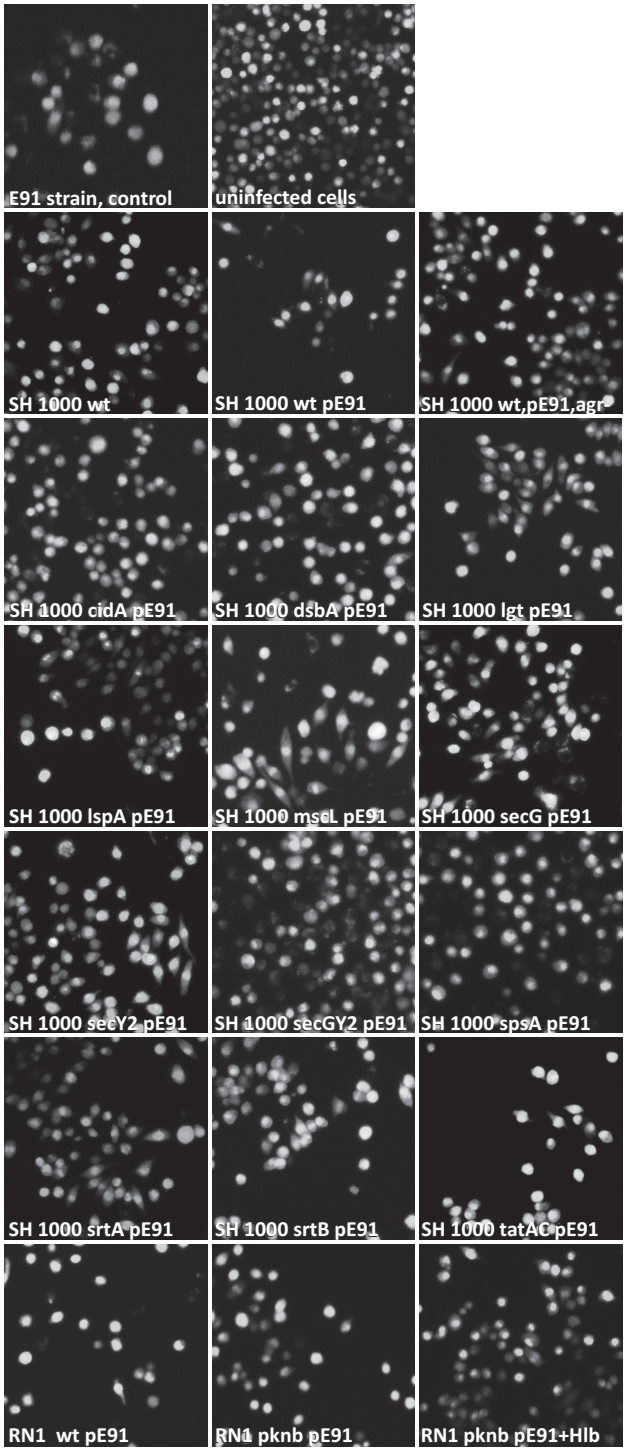


Figure 2. Secretion of penicillinase into J774 macrophages by different strains of *S. aureus*. The secretion of an active penicillinase by wild-type *S. aureus* or mutant *S. aureus* strains with different protein secretion defects was assessed by fluorescence microscopy. For this purpose, the infected macrophages were loaded with CCF2-AM. Cells in which intracellular secretion of penicillinase has occurred appear blue.

Chapter 7

Nederlandse samenvatting

Staphylococcus aureus is een van de meest voorkomende en veelzijdige menselijke ziekteverwekkers. Deze bacterie is verantwoordelijk voor een breed spectrum van besmettelijke ziekten, die in ernst variëren van zeer mild tot dodelijk. Ongeveer 30% van de menselijke bevolking is asymptomatisch gekoloniseerd met *S. aureus*, met name op de huid, in de bovenste luchtwegen en in de darmen. Bij sommige individuen veroorzaakt deze bacterie echter ernstige invasieve infecties en dit is de reden waarom *S. aureus* zo berucht is als ziekteverwekker.

S. aureus is een 'succesvolle' ziekteverwekker door het uitscheiden van een groot arsenaal aan virulentiefactoren, die de bacterie kan aangemaken als de omstandigheden hiertoe aanleiding geven. Sommige uitgescheiden virulentiefactoren blijven aan het celoppervlak hangen. Deze oppervlakte-eiwitten helpen de bacterie bij de gastheerkolonisatie. Andere virulentiefactoren, zoals invasines en proteases, zorgen voor de verspreiding van de bacterie in de gastheer. Meerdere toxines kunnen gastheercellen beschadigen, waardoor de natuurlijk barrieres tegen infectie doorbroken worden. Recent onderzoek heeft extra aandacht gevestigd op een serine/threonine kinase van *S. aureus*, PknB genaamd. PknB is een enzym dat andere eiwitten kan fosforyleren en dergelijke enzymen staan bekend als kinases. PknB is met name opgevallen doordat het sterk lijkt op soortgelijke kinases van eukaryote organismen waaronder de mens. In *S. aureus* blijkt PknB betrokken te zijn bij het bacteriële metabolisme en de celwandsynthese. De rol die PknB speelt in de pathogenese van *S. aureus* was nog niet opgehelderd toen de studies beschreven in dit proefschrift gestart werden. Aangezien PknB behoort tot een serine/ threonine kinase-type, dat betrokken is bij de virulentie van verschillende andere bacteriële ziekteverwekkers zoals bijvoorbeeld *Yersinia pestis* - de veroorzaker van de pest - werd besloten om de rol van PknB in de interacties tussen *S. aureus* en immuuncellen van de gastheer nader te onderzoeken.

Pathogene micro-organismen worden in eerste instantie herkend door cellen van het immuunsysteem met behulp van zogenaamde patroonherkenningsreceptoren (PRRS), zoals de Toll-like receptoren (TLR's) en de Nod-receptoren. De meeste bacteriën worden herkend door meer dan één receptor en deze receptoren kunnen bovendien ook nog interacties aangaan met elkaar. Hierdoor leidt de activatie van dergelijke receptoren tot de activatie van een complex netwerk van signaaloverdrachtsreacties, waarbij ook nog communicatie

optreedt tussen de verschillende geactiveerde 'stroomafwaarts'-gelegen signaalmoleculen. In **hoofdstuk 2** is de ontrafeling van het netwerk van complexe interacties tussen *S. aureus* en menselijke macrofagen beschreven. Op basis van de resultaten wordt een algemeen overzicht van de interacties tussen deze gastheercellen en *S. aureus* gepresenteerd.

Met behulp van de 'kinase profiling' techniek, die gebruik maakt van peptide microarrays, is zichtbaar gemaakt dat de reacties van macrofagen op de aanwezigheid van *S. aureus* worden gemedieerd door meerdere receptoren, waaronder de zogenaamde G-protein coupled receptor, receptor tyrosine kinases, de scavenger receptor 36 en integrines. Deze resultaten stellen het klassieke concept, dat bacteriële ontstekingsreacties in hoofdzaak worden geactiveerd door middel van Toll-like receptor 2 en NF-kB-signalering ter discussie. Bovendien laten de resultaten ook zien, dat zogenaamde Mitogen-activated protein kinases, JNK in het bijzonder, een belangrijke rol spelen in het orkestreren van de afweer bij een *S. aureus*-infectie. Om te onderzoeken hoe mogelijke interacties tussen *S. aureus* en de humane macrofagen plaatsvinden werd voor een proteomics benadering gekozen. Door middel van gel-vrije proteoom analyses op niet-gefagocyteerd *S. aureus* cellen werd een aantal *S. aureus*-gebonden eiwitten geïdentificeerd, die van de macrofagen afkomstig zijn. Deze omvatten heat shock eiwitten, antimicrobiële histonen, enolase en diverse andere eiwitten. Sommige van deze eiwitten zijn onderdelen van 'extracellulaire vallen', die worden gevormd door fagocyten na stimulatering met mitogenen, cytokines of de ziekteverwekkers zelf. De proteomics analyses lieten ook zien, dat *S. aureus* na 1 uur van incubatie met menselijke macrofagen een stress reactie vertoont, die typerend is voor condities van voedsel-limitatie. Deze 'stringent response' wordt onder andere gekenmerkt door een sterke afname van eiwitten die betrokken zijn bij de eiwitsynthese en een gelijktijdige verhoging van de eiwitten die verantwoordelijk zijn voor de celstofwisseling. De resultaten lieten ook een zogenaamde σ^B-afhankelijke algemene stress reactie in de *S. aureus* cellen zien, zoals weerspiegeld door verhoogde concentraties van de RsbV en RsbW eiwitten en subeenheden van de ATP-afhankelijke Clp protease. Interessant is dat de proteomics data ook aantonen dat *S. aureus* cellen, gekweekt in de aanwezigheid van macrofagen, de serine/threonine kinase PknB produceerden, terwijl dit eiwit is niet detecteerbaar was in *S. aureus* cellen die geïncubeerd werden zonder macrofagen.

Hoewel eerder is aangetoond dat Pknb betrokken is bij het celwandmetabolisme van *S. aureus* en de resistentie tegen antibiotica, was nog niet eerder onderzocht of PknB menselijke eiwitten kan fosforyleren. Daarom werden dezelfde peptide microarrays, die in hoofdstuk 2 ook gebruikt zijn voor kinase profiling, nu ook gebruikt om de specificiteit van gezuiverd PknB definiëren. Dit was een relevante benadering, omdat full-size PknB aanwezig blijkt te zijn in het groeimedium van *S. aureus* cellen. Dit suggereert immers dat PknB doeleiwitten in immuuncellen van de gastheer zou kunnen fosforyleren, in het bijzonder na de internalisatie van *S. aureus* door middel van fagocytose. De resultaten van deze analyses zijn gedocumenteerd in **hoofdstuk 3**, waar gezuiverd PknB werd gebruikt voor kinase profiling in plaats van de eerder gebruikte lysaten van macrofagen. De resultaten tonen aan dat PknB inderdaad bekende substraten van menselijke serine/threonine kinases kan herkennen en fosforyleren. Bovendien is aangetoond dat proline een onderdeel is van het PknB-herkenningsmotief. Dit betekent dat PknB behoort tot de familie van proline-gerichte kinases, die bijvoorbeeld ook de 'cycline-afhankelijke protein kinases', het 'glycogen synthase kinase-3' en de 'mitogen-activated protein kinases' (MAP kinases) omvat. De studies beschreven in **hoofdstuk 3** suggereren derhalve dat actief PknB kan vrijkomen uit invasieve *S. aureus* cellen en dat dit enzym kan participeren in de subversie van de gastheercel door de signaaltrans-ductiemechanismen van de gastheer te beïnvloeden. Daarom werd de rol van *S. aureus* PknB in de infectie van humane perifere bloed mononucleaire cellen (PBMC's) en macrofagen bestudeerd met behulp van 'enzym-linked immune assays' (ELISA) en kinase profiling. De resultaten gepresenteerd in **hoofdstuk 4** laten zien, dat infectie van menselijke PBMCs met *S. aureus* zonder het *pknB* gen resulteert in een sterk verminderde secretie van ontstekingsmediatoren, zoals interleukine 1β (IL-1β), interleukine 6 (IL-6), interleukine 8 (IL-8), interleukine-10 (IL-10) en tumor necrose factor α (TNF -α). Om verder te onderzoeken of de verminderde cytokineproductie kan worden gecorreleerd met een verminderde activiteit van gastheerkinasen is opnieuw de kinase profiling assay gebruikt. De resultaten van kinase profiling laten zien, dat de aanwezigheid van PknB-deficiënte *S. aureus* cellen resulteert in een verminderde activiteit van belangrijke pro-inflammatoire kinasen, zoals de Bruton's tyrosine kinase, de Focal adhesion kinase en kinases van de Src-familie. Daarnaast laten de resultaten gepresenteerd in **hoofdstuk 4** zien, dat het bacteriofaag 13 de intensiteit van de

macrofaagsignaaltransductiereacties op PknB-deficiënte *S. aureus* cellen sterk beperkt. Dit bacteriofaag speelt een belangrijke rol in de verdediging van *S. aureus* tegen de humane afweer, omdat het de genen draagt voor CHIPS (Chemotaxis Inhibiting Protein of *S. aureus*), SCIN (*Staphylococcus* complement inhibitor) en staphylokinase - drie eiwitten die van belang zijn bij de ontwijking van het humane immuunsysteem. In **hoofdstuk 4** zijn drie mogelijke scenario's voorgesteld om de waargenomen vermindering in de productie van ontstekingsmediatoren door PBMC's en de downregulatie van tyrosine kinases in macrofagen geïncubeerd met *S. aureus* zonder *pknB* uit te leggen. In de eerste plaats, zou het zo kunnen zijn, dat uitgescheiden PknB van geïnternaliseerde *S. aureus* cellen bepaalde gastheereiwitten fosforyleert (zie hoofdstuk 3) en daardoor direct invloed uitoefent op de tyrosine kinase signaaltransductie. Echter, de resultaten beschreven in hoofdstuk 3 geven niet aan dat PknB de geïdentificeerde targets van tyrosine kinases herkent. Daarom is het moeilijk om te concluderen of PknB rechtstreeks van invloed is op de signaaltransductie via tyrosine kinases. PknB heeft een proline-gerichte serine/threonine kinase activiteit, die vergelijkbare is met de activiteit van de eukaryote MAP-kinases (hoofdstuk 3). Bovendien laten de verkregen resultaten zien, dat PknB de Activating transcription factor-2 (ATF-2) - de natuurlijke target van MAP kinases - kan fosforyleren. Het zal echter moeilijk zijn om aan te tonen of dit ook echt *in vivo* plaatsvindt, omdat ATF-2 in zeer sterke mate gefosforyleerd wordt door MAP-kinases. Het blijft dan ook een grote uitdaging om onderscheid te maken tussen de fosforylering gemedieerd door MAP-kinases en PknB. Hetzelfde geldt voor de fosforylering van andere eiwitten, waarvan via kinase profiling werd aangetoond dat PknB ze kan herkennen. Een tweede verklaring voor de verminderde ontstekingsreacties tengevolge van PknB-deficiënte *S. aureus* cellen, is de verminderde activiteit van PknB-gereguleerde autolysines en de verminderde turnover van de *S. aureus* cellen. Een verminderde celwand turnover zou kunnen resulteren in het vrijkomen van lagere hoeveelheden peptidoglycaan van de bacteriën. Op zijn beurt kan dit invloed hebben op de herkenning van het bacteriële peptidoglycaan of de gehele bacteriën door macrofagen en PBMC's. Een derde reden voor de verminderde respons van immuuncellen op PknB-defieciënte *S. aureus* cellen zou een mogelijke structurele verandering in het bacteriële peptidoglycaan kunnen zijn, die veroorzaakt wordt door de afwezigheid van PknB. Deze verandering zou bijvoorbeeld van invloed kunnen zijn op bepaalde

peptidoglycaan-lipoteichoïnezuur interacties welke verantwoordelijk zijn voor de juiste herkenning van *S. aureus* en aanleiding geven tot het ontstaan van ontstekingsreacties. Ten slotte blijkt uit de huidige gegevens duidelijk dat *S. aureus* PknB nodig heeft om een sterke ontsteking te provoceren. Daarom is het goed voorstelbaar dat specifieke PknB remmers, die nog ontwikkeld moeten worden, in de toekomst toepassingen vinden in de bestrijding van de fulminante pathologie van *S. aureus* infecties.

Een belangrijke resterende vraag betrof de rol van PknB in de intracellulaire overleving van *S. aureus* na fagocytose. In **hoofdstuk 5** wordt het gebruik van een assay beschreven om de uitscheiding van een penicillinase door *S. aureus* cellen te bestuderen na hun internalisatie in macrofagen van muizen. Voor dit doel werd een β-lactamase activiteitstest toegepast, waarmee penicillinase secretie door geïnternaliseerde macrofagen via fluorescentiemicroscopie bestudeerd kan worden. Met behulp van deze test werd de rol van verschillende componenten van de bacteriële eiwitsecretiemachinerie en de secretie van PknB onderzocht na opname van *S. aureus* door de macrofagen. De resultaten laten een verhoogde penicillinase activiteit zien in macrofagen, die mutante *S. aureus* cellen hebben opgenomen waarin de diacyl-glyceryl transferase Lgt, de thiol-disulfide oxidoreductase DsbA, het mechanosensitieve kanaal MscL of de eiwittranslocasecomponenten SecG en SecY2 ontbreken. Dit suggereert dat de afwezigheid van LGT, DsbA, MscL of SecG/SecY2 resulteert in (i) een verbeterde intracellulaire overleving van de mutante *S. aureus* bacteriën, (ii) in verhoogde niveaus van penicillinase secretie door *S. aureus* in de macrofagen, of (iii) beide. Een zeer belangrijke waarneming was, dat de afwezigheid van PknB niet meetbaar van invloed was op de penicillinase activiteit, die in de macrofagen waargenomen werd. Dit impliceert dat de overleving van PknB-deficiënte *S. aureus* cellen in muizenmacrofagen niet verminderd is ten opzichte van de overleving van PknB-proficiënte *S. aureus* cellen. Bij elkaar genomen bieden de in dit proefschrift beschreven studies een overzicht van het complexe netwerk van signaaltransductiereacties, die zich ontvouwen als de pathogene *S. aureus* bacterie in conflict komt met immuuncellen van de gastheer.

Chapter 8

Summary in Polish /

Podsumowanie w języku polskim

Gronkowiec złocisty (łac. *Staphylococcus aureus*) jest Gram-dodatnią bakterią, która należy do najbardziej chorobotwórczych gatunków wśród bakterii gronkowca powodujących choroby skóry, zapalenie płuc jak i również zatrucia pokarmowe. Gronkowiec złocisty występuje w jamie nosowo-gardłowej oraz na skórze ludzi i zwierząt. Szacuje się, że od 10% do 50% populacji ludzkiej stale lub okresowo jest nosicielami tych bakterii bez występowania objawów chorobowych. Jednak zdarza się, że u osób z osłabioną odpornością zakażenie gronkowcem złocistym może prowadzić do wielu powikłań, takich jak zapalenie mięśnia sercowego, zapalenie kości czy też posocznicy gronkowcowej.

Gronkowiec złocisty swoją efektywność w wywoływaniu różnych chorób zawdzięcza arsenałowi czynników wirulencji, które są produkowane przez gronkowca w różnych warunkach i na różnych etapach rozwoju bakterii. Do czynników wirulencji należą białka powierzchniowe pomagające w kolonizacji gronkowca, inwazyny i proteazy - białka powodujące rozprzestrzenianie się bakterii oraz czynniki powierzchniowe - takie jak białko A (ang. staphylococcal protein A, SpA). Białko A odpowiedzialne jest za wiązanie immunoglobuliny IgG poprzez region Fc, czym co zapobiega opsonizacji i fagocytozie bakterii. Ponadto, gronkowiec produkuje wiele toksyn, które poprzez działanie na błonę komórkową zabijają komórki układu immunologicznego.

Najnowsze badania naukowe coraz częściej koncentrują się na bakteryjnych kinazach serynowo-treoninowych, czyli białkach odpowiedzialnych za wiele procesów życiowych bakterii. Kinazy te biorą one udział w metabolizmie, syntezie ściany komórkowej, a także patogenezie. Gronkowiec złocisty podobnie jak inne patogeny, również posiada kinazę serynowo-treoninową o nazwie PknB. Uważa się, że PknB odgrywa ważną rolę w patogenezie gronkowca złocistego, jednak dokładna jej fukcja nie została jeszcze wyjaśniona.

Celem tej pracy było zbadanie i opisanie interakcji pomiędzy gronkowcem złocistym i fagocytami (komórkami żernymi), a także wyjaśnienie funkcji kinazy PknB w patogenezie *S. aureus*.

Zanim chorobotwórcze mikroorganizmy zostaną pochłonięte i zabite przez komórki układu odpornościowego, są najpierw przez nie rozpoznawane za pomocą receptorów. Komórki rozpoznają bakteryjne struktury np. peptydoglikany czy lipoproteiny poprzez receptory (PRRS). Do receptorów, które biorą udział w rozpoznawaniu bakterii możemy zaliczyć Toll-like receptory (TLRs), receptory

sprzężone z białkami G (GPCR), receptory NOD, czy Fc receptory. Bakteryjne struktury mogą być jednak rozpoznane przez więcej niż jeden receptor i dodatkowo receptory te mogą również wchodzić w interakcje między sobą. Pobudzenie różnych receptorów i ich interakcje prowadzą do aktywacji wielu szlaków sygnałowych w komórkach żernych, mających na celu unieszkodliwienie bakterii.

W rozdziale 2 przedstawione są interakcje pomiędzy gronkowcem i ludzkimi makrofagami, a także ogólny przegląd interakcji pomiędzy komórkami ludzkimi i białkami produkowanymi przez gronkowca złocistego. W celu rozwikłania tych skomplikowanych interakcji wykorzystane zostały makromacierze z peptydami tzw. pepchipy. Pepchip, to płytka szklana z naniesionymi peptydami (ok. 11 amino kwasów), które są rozpoznawane przez kinazy aktywowane podczas infekcji gronkowcem. Profilowanie kinaz w rozdziale 2, wykazało, że *S. aureus* jest rozpoznawany przez wiele receptorów, w tym receptory sprzężone z białkami G (GPCR), receptorowe kinazy tyrozynowe, Scavenger receptor 36, i integryny. Jednocześnie wyniki kwestionują klasyczną koncepcję, że odpowiedź zapalna podczas infekcji *S. aureus* jest aktywowana głównie przez Toll-like receptor 2 i sygnalizację poprzez NF-kB. Ponadto wyniki pokazują również, że kinazy aktywowane mitogenami (MAP kinazy), w szczególności kinaza JNK, odgrywają ważną rolę w odpowiedzi immunologicznej podczas infekcji gronkowcem złocistym. Dodatkowo w celu zbadania wzajemnych oddziaływań pomiędzy *S. aureus* i ludzkimi makrofagami zastosowana została spektrometria masowa, za pomocą której zostały zidentyfikowane różne klasy białek ludzkich związanych z gronkowcem. Wśród tych białek można wyróżnić Białka szoku cieplnego (ang. Heat shock proteins), przeciwbakteryjne histony, enolaza oraz inne białka.

W rozdziale 3 analizie zostały poddane właściwości kinazy PknB z *S. aureus* i jej zdolności do fosforylowania ludzkich białek. Wyniki wykazały, że oczyszczona kinaza PknB rozpoznaje i fosforyluje ludzkie peptydy naniesione na pepchipy). Dodatkowo wykazano, że prolina jest częścią sekwencji rozpoznawaną przez PknB. Dlatego też, badania opisane w rozdziale 3 sugerują, że aktywna kinaza PknB uwolniona przez gronkowca znajdującego się wewnątrz makrofaga podczas infekcji, może rozpoznać i ufosforylować ludzkie białka. To z kolei prowadzić może do całkowitej zmiany transdukcji sygnału w komórce gospodarza, braku prawidłowej odpowiedzi immunologicznej i w rezultacie do przeżycia gronkowca wewnątrz fagocytów. Wyniki przedstawione w rozdziale 4 wskazują natomiast,

że zakażenie ludzkich jednojądrzastych komórek krwi obwodowej (PBMC) gronkowcem nie posiadającym kinazy PknB prowadzi do zmniejszonej sekrecji mediatorów zapalnych, takich jak interleukiny 1β (IL-1β) i interleukiny 6 (IL-6), interleukiny 8 (IL-8), interleukiny 10 (IL- 10) i czynnika martwicy nowotworów α (TNF-α). Ponadto, w celu dalszego zbadania, czy zmniejszona ilość cytokin wytwarzanych przez PBMC może być skorelowana ze zmniejszoną aktywnością kinaz, zostały użyte pepchipy (Podobnie jak w Rozdziale 2 i 3). Zgodnie z postawioną hipotezą, wyniki profilowania kinaz wykazały zmniejszoną aktywność prozapalnych kinaz takich jak kinaza Brutona (Btk) oraz kinaza Src, co prawdopodobnie niekorzystnie wpływa na produkcję prozapalnych cytokin.

Kolejnym zagadnieniem, któremu poświęcony został Rozdział 5, jest funkcja kinazy PknB w wewnątrzkomórkowym przetrwaniu *S. aureus* podczas fagocytozy. W tym celu zbadana została sekrecja penicylinazy przez komórki *S. aureus*. Wyniki pokazują, że zmutowane komórki gronkowca, którym brakuje genów *lgt*, *dsbA, mscL* lub *secG* i *secY2,* wykazywały większą aktywność penicylinazy niż macierzysty szczep gronkowca. Powyższe wyniki sugerują zatem, że brak diacylglyceryl transferazy (Lgt), tiol-dwusiarczku-oksydoreduktazy (DsbA), mechano-sensytywnego kanału MscL lub SecG i SecY2 pozytywnie wpływa na wewnątrzkomórkowe przetrwanie zmutowanych komórek gronkowca złocistego. Co ważne, brak PknB nie ma wpływu na przeżywalność *S. aureus* wewnątrz makrofagów.

Podsumowując, obecne badania po raz pierwszy wnikliwie opisują skomplikowaną sieć interakcji, które mają miejsce w komórkach odpornościowych podczas infekcji gronkowcem złocistym.

Chapter 9

References

1. Miller M, Dreisbach A, Otto A, Becher D, Bernhardt J, et al. Mapping of Interactions between Human Macrophages and Staphylococcus aureus Reveals an Involvement of MAP Kinase Signaling in the Host Defense. J Proteome Res.
2. Deftereos SP, Michailidou E, Karagiannakis GK, Grigoriadi S, Prassopoulos P (2009) Hematogenous infantile infection presenting as osteomyelitis and septic arthritis: a case report. Cases J 2: 8293.
3. Geipel U (2009) Pathogenic organisms in hip joint infections. Int J Med Sci 6: 234-240.
4. Carrillo-Marquez MA, Hulten KG, Hammerman W, Mason EO, Kaplan SL (2009) USA300 is the Predominant Genotype Causing Staphylococcus aureus Septic Arthritis in Children. Pediatr Infect Dis J.
5. Vollmer W, Blanot D, de Pedro MA (2008) Peptidoglycan structure and architecture. FEMS Microbiol Rev 32: 149-167.
6. Wang JE, Jorgensen PF, Almlof M, Thiemermann C, Foster SJ, et al. (2000) Peptidoglycan and lipoteichoic acid from Staphylococcus aureus induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. Infect Immun 68: 3965-3970.
7. Matsui K, Wirotesangthong M, Nishikawa A (2008) Peptidoglycan from Staphylococcus aureus induces IL-4 production from murine spleen cells via an IL-18-dependent mechanism. Int Arch Allergy Immunol 146: 262-266.
8. Garver LS, Wu J, Wu LP (2006) The peptidoglycan recognition protein PGRP-SC1a is essential for Toll signaling and phagocytosis of Staphylococcus aureus in Drosophila. Proc Natl Acad Sci U S A 103: 660-665.
9. Bera A, Herbert S, Jakob A, Vollmer W, Gotz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol 55: 778-787.
10. Lee CY (1992) Cloning of genes affecting capsule expression in Staphylococcus aureus strain M. Mol Microbiol 6: 1515-1522.
11. Lee CY, Lee JC (2006) Staphylococcal capsule. In: Fischetti VA, Novick, R.P, Ferretti, J.J, Portnoy,D.A, Rood,J.I, editor. Gram-positive pathogens 2 ed. Washington, D.C: ASM Press. pp. 456-463.
12. O'Riordan K, Lee JC (2004) Staphylococcus aureus capsular polysaccharides. Clin Microbiol Rev 17: 218-234.
13. Scott JR, Barnett TC (2006) Surface proteins of gram-positive bacteria and how they get there. Annu Rev Microbiol 60: 397-423.
14. Kampen AH, Tollersrud T, Lund A (2005) Staphylococcus aureus capsular polysaccharide types 5 and 8 reduce killing by bovine neutrophils in vitro. Infect Immun 73: 1578-1583.
15. Watts A, Ke D, Wang Q, Pillay A, Nicholson-Weller A, et al. (2005) Staphylococcus aureus strains that express serotype 5 or serotype 8 capsular polysaccharides differ in virulence. Infect Immun 73: 3502-3511.
16. Cunnion KM, Hair PS, Buescher ES (2004) Cleavage of complement C3b to iC3b on the surface of Staphylococcus aureus is mediated by serum complement factor I. Infect Immun 72: 2858-2863.
17. Baddiley J (1972) Teichoic acids in cell walls and membranes of bacteria. Essays Biochem 8: 35-77.
18. Weidenmaier C, Peschel A (2008) Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Rev Microbiol 6: 276-287.
19. Neuhaus FC, Baddiley J (2003) A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev 67: 686-723.
20. Tomasz A, Westphal M, Briles EB, Fletcher P (1975) On the physiological functions of teichoic acids. J Supramol Struct 3: 1-16.
21. Weidenmaier C, Kokai-Kun JF, Kristian SA, Chanturiya T, Kalbacher H, et al. (2004) Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections. Nat Med 10: 243-245.
22. D'Elia MA, Millar KE, Beveridge TJ, Brown ED (2006) Wall teichoic acid polymers are dispensable for cell viability in Bacillus subtilis. J Bacteriol 188: 8313-8316.
23. Weidenmaier C, Peschel A, Xiong YQ, Kristian SA, Dietz K, et al. (2005) Lack of wall teichoic acids in Staphylococcus aureus leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. J Infect Dis 191: 1771-1777.
24. Weidenmaier C, Kokai-Kun JF, Kulauzovic E, Kohler T, Thumm G, et al. (2008) Differential roles of sortase-anchored surface proteins and wall teichoic acid in Staphylococcus aureus nasal colonization. Int J Med Microbiol 298: 505-513.
25. Tzianabos AO, Wang JY, Lee JC (2001) Structural rationale for the modulation of abscess formation by Staphylococcus aureus capsular polysaccharides. Proc Natl Acad Sci U S A 98: 9365-9370.
26. Weidenmaier C, McLoughlin RM, Lee JC (2010) The zwitterionic cell wall teichoic acid of Staphylococcus aureus provokes skin abscesses in mice by a novel CD4+ T-cell-dependent mechanism. PLoS One 5: e13227.
27. Xia G, Kohler T, Peschel A (2010) The wall teichoic acid and lipoteichoic acid polymers of Staphylococcus aureus. Int J Med Microbiol 300: 148-154.
28. Majcherczyk PA, Rubli E, Heumann D, Glauser MP, Moreillon P (2003) Teichoic acids are not required for Streptococcus pneumoniae and Staphylococcus aureus cell walls to trigger the release of tumor necrosis factor by peripheral blood monocytes. Infect Immun 71: 3707-3713.
29. Atilano ML, Yates J, Glittenberg M, Filipe SR, Ligoxygakis P (2011) Wall Teichoic Acids of Staphylococcus aureus Limit Recognition by the Drosophila Peptidoglycan Recognition Protein-SA to Promote Pathogenicity. PLoS Pathog 7: e1002421.
30. Kengatharan KM, De Kimpe S, Robson C, Foster SJ, Thiemermann C (1998) Mechanism of gram-positive shock: identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. J Exp Med 188: 305-315.
31. Bhakdi S, Klonisch T, Nuber P, Fischer W (1991) Stimulation of monokine production by lipoteichoic acids. Infect Immun 59: 4614-4620.
32. Cleveland MG, Gorham JD, Murphy TL, Tuomanen E, Murphy KM (1996) Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect Immun 64: 1906-1912.
33. Danforth JM, Strieter RM, Kunkel SL, Arenberg DA, VanOtteren GM, et al. (1995) Macrophage inflammatory protein-1 alpha expression in vivo and in vitro: the role of lipoteichoic acid. Clin Immunol Immunopathol 74: 77-83.
34. Keller R, Fischer W, Keist R, Bassetti S (1992) Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect Immun 60: 3664-3672.

35. Standiford TJ, Arenberg DA, Danforth JM, Kunkel SL, VanOtteren GM, et al. (1994) Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. Infect Immun 62: 119-125.
36. von Aulock S, Morath S, Hareng L, Knapp S, van Kessel KP, et al. (2003) Lipoteichoic acid from Staphylococcus aureus is a potent stimulus for neutrophil recruitment. Immunobiology 208: 413-422.
37. Heilmann C (2011) Adhesion mechanisms of staphylococci. Adv Exp Med Biol 715: 105-123.
38. Elasri MO, Thomas JR, Skinner RA, Blevins JS, Beenken KE, et al. (2002) Staphylococcus aureus collagen adhesin contributes to the pathogenesis of osteomyelitis. Bone 30: 275-280.
39. Lee LY, Hook M, Haviland D, Wetsel RA, Yonter EO, et al. (2004) Inhibition of complement activation by a secreted Staphylococcus aureus protein. J Infect Dis 190: 571-579.
40. Sasso EH, Silverman GJ, Mannik M (1991) Human IgA and IgG F(ab')2 that bind to staphylococcal protein A belong to the VHIII subgroup. J Immunol 147: 1877-1883.
41. Silverman GJ, Goodyear CS, Siegel DL (2005) On the mechanism of staphylococcal protein A immunomodulation. Transfusion 45: 274-280.
42. Palmqvist N, Foster T, Tarkowski A, Josefsson E (2002) Protein A is a virulence factor in Staphylococcus aureus arthritis and septic death. Microb Pathog 33: 239-249.
43. Patel AH, Nowlan P, Weavers ED, Foster T (1987) Virulence of protein A-deficient and alpha-toxin-deficient mutants of Staphylococcus aureus isolated by allele replacement. Infect Immun 55: 3103-3110.
44. Goward CR, Scawen MD, Murphy JP, Atkinson T (1993) Molecular evolution of bacterial cell-surface proteins. Trends Biochem Sci 18: 136-140.
45. Atkins KL, Burman JD, Chamberlain ES, Cooper JE, Poutrel B, et al. (2008) *S. aureus* IgG-binding proteins SpA and Sbi: host specificity and mechanisms of immune complex formation. Mol Immunol 45: 1600-1611.
46. Zhang L, Jacobsson K, Vasi J, Lindberg M, Frykberg L (1998) A second IgG-binding protein in Staphylococcus aureus. Microbiology 144 (Pt 4): 985-991.
47. Zhang L, Jacobsson K, Strom K, Lindberg M, Frykberg L (1999) Staphylococcus aureus expresses a cell surface protein that binds both IgG and beta2-glycoprotein I. Microbiology 145 (Pt 1): 177-183.
48. Burman JD, Leung E, Atkins KL, O'Seaghdha MN, Lango L, et al. (2008) Interaction of human complement with Sbi, a staphylococcal immunoglobulin-binding protein: indications of a novel mechanism of complement evasion by Staphylococcus aureus. J Biol Chem 283: 17579-17593.
49. Rooijakkers SH, van Wamel WJ, Ruyken M, van Kessel KP, van Strijp JA (2005) Anti-opsonic properties of staphylokinase. Microbes Infect 7: 476-484.
50. Rooijakkers SH, Ruyken M, Roos A, Daha MR, Presanis JS, et al. (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol 6: 920-927.
51. Garcia BL, Tzekou A, Ramyar KX, McWhorter WJ, Ricklin D, et al. (2009) Crystallization of human complement component C3b in the presence of a staphylococcal complement-inhibitor protein (SCIN). Acta Crystallogr Sect F Struct Biol Cryst Commun 65: 482-485.
52. van Wamel WJ, Rooijakkers SH, Ruyken M, van Kessel KP, van Strijp JA (2006) The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of Staphylococcus aureus are located on beta-hemolysin-converting bacteriophages. J Bacteriol 188: 1310-1315.
53. Postma B, Poppelier MJ, van Galen JC, Prossnitz ER, van Strijp JA, et al. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus binds specifically to the C5a and formylated peptide receptor. J Immunol 172: 6994-7001.
54. de Haas CJ, Veldkamp KE, Peschel A, Weerkamp F, Van Wamel WJ, et al. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. J Exp Med 199: 687-695.
55. Palma M, Shannon O, Quezada HC, Berg A, Flock JI (2001) Extracellular fibrinogen-binding protein, Efb, from Staphylococcus aureus blocks platelet aggregation due to its binding to the alpha-chain. J Biol Chem 276: 31691-31697.
56. Heilmann C, Herrmann M, Kehrel BE, Peters G (2002) Platelet-binding domains in 2 fibrinogen-binding proteins of Staphylococcus aureus identified by phage display. J Infect Dis 186: 32-39.
57. Shannon O, Uekotter A, Flock JI (2005) Extracellular fibrinogen binding protein, Efb, from Staphylococcus aureus as an antiplatelet agent in vivo. Thromb Haemost 93: 927-931.
58. Lee LY, Liang X, Hook M, Brown EL (2004) Identification and characterization of the C3 binding domain of the Staphylococcus aureus extracellular fibrinogen-binding protein (Efb). J Biol Chem 279: 50710-50716.
59. Hess DJ, Henry-Stanley MJ, Erickson EA, Wells CL (2003) Intracellular survival of Staphylococcus aureus within cultured enterocytes. J Surg Res 114: 42-49.
60. Ellington JK, Reilly SS, Ramp WK, Smeltzer MS, Kellam JF, et al. (1999) Mechanisms of Staphylococcus aureus invasion of cultured osteoblasts. Microb Pathog 26: 317-323.
61. Priest DH, Peacock JE, Jr. (2005) Hematogenous vertebral osteomyelitis due to Staphylococcus aureus in the adult: clinical features and therapeutic outcomes. South Med J 98: 854-862.
62. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, et al. (2005) Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils. J Immunol 175: 3907-3919.
63. Das D, Saha SS, Bishayi B (2008) Intracellular survival of Staphylococcus aureus: correlating production of catalase and superoxide dismutase with levels of inflammatory cytokines. Inflamm Res 57: 340-349.
64. Schnaith A, Kashkar H, Leggio SA, Addicks K, Kronke M, et al. (2007) Staphylococcus aureus subvert autophagy for induction of caspase-independent host cell death. J Biol Chem 282: 2695-2706.
65. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, et al. (2000) Survival of Staphylococcus aureus inside neutrophils contributes to infection. J Immunol 164: 3713-3722.
66. Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, et al. (2008) A potential new pathway for Staphylococcus aureus dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. PLoS ONE 3: e1409.
67. Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2: 675-680.

68. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388: 394-397.
69. Dziarski R, Wang Q, Miyake K, Kirschning CJ, Gupta D (2001) MD-2 enables Toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to Gram-positive and Gram-negative bacteria and their cell wall components. J Immunol 166: 1938-1944.
70. Heine H, Kirschning CJ, Lien E, Monks BG, Rothe M, et al. (1999) Cutting edge: cells that carry A null allele for toll-like receptor 2 are capable of responding to endotoxin. J Immunol 162: 6971-6975.
71. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 274: 17406-17409.
72. Bubeck Wardenburg J, Williams WA, Missiakas D (2006) Host defenses against Staphylococcus aureus infection require recognition of bacterial lipoproteins. Proc Natl Acad Sci U S A 103: 13831-13836.
73. Stoll H, Dengjel J, Nerz C, Gotz F (2005) Staphylococcus aureus deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. Infect Immun 73: 2411-2423.
74. Hashimoto M, Tawaratsumida K, Kariya H, Kiyohara A, Suda Y, et al. (2006) Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in Staphylococcus aureus. J Immunol 177: 3162-3169.
75. Han SH, Kim JH, Martin M, Michalek SM, Nahm MH (2003) Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2. Infect Immun 71: 5541-5548.
76. Bulut Y, Faure E, Thomas L, Equils O, Arditi M (2001) Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. J Immunol 167: 987-994.
77. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, et al. (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285: 736-739.
78. Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, et al. (1999) Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285: 732-736.
79. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, et al. (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 13: 933-940.
80. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, et al. (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 169: 10-14.
81. Sandor F, Latz E, Re F, Mandell L, Repik G, et al. (2003) Importance of extra- and intracellular domains of TLR1 and TLR2 in NFkappa B signaling. J Cell Biol 162: 1099-1110.
82. Nilsen NJ, Deininger S, Nonstad U, Skjeldal F, Husebye H, et al. (2008) Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling: role of CD14 and CD36. J Leukoc Biol 84: 280-291.
83. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249: 1431-1433.
84. Jorgensen PF, Wang JE, Almlof M, Thiemermann C, Foster SJ, et al. (2001) Peptidoglycan and lipoteichoic acid modify monocyte phenotype in human whole blood. Clin Diagn Lab Immunol 8: 515-521.
85. Takeuchi O, Hoshino K, Akira S (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J Immunol 165: 5392-5396.
86. Chen G, Shaw MH, Kim YG, Nunez G (2009) NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol 4: 365-398.
87. Deshmukh HS, Hamburger JB, Ahn SH, McCafferty DG, Yang SR, et al. (2009) Critical role of NOD2 in regulating the immune response to Staphylococcus aureus. Infect Immun 77: 1376-1382.
88. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G (2009) The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol 10: 241-247.
89. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, et al. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol 9: 847-856.
90. Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, et al. (2007) Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. Immunity 26: 433-443.
91. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 440: 237-241.
92. Miller LS, Pietras EM, Uricchio LH, Hirano K, Rao S, et al. (2007) Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against Staphylococcus aureus in vivo. J Immunol 179: 6933-6942.
93. Miller LS, O'Connell RM, Gutierrez MA, Pietras EM, Shahangian A, et al. (2006) MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against Staphylococcus aureus. Immunity 24: 79-91.
94. Munoz-Planillo R, Franchi L, Miller LS, Nunez G (2009) A critical role for hemolysins and bacterial lipoproteins in Staphylococcus aureus-induced activation of the Nlrp3 inflammasome. J Immunol 183: 3942-3948.
95. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440: 228-232.
96. Dziarski R, Gupta D (2010) Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity. Innate Immun 16: 168-174.
97. Royet J, Gupta D, Dziarski R (2011) Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. Nat Rev Immunol 11: 837-851.
98. Shah IM, Laaberki MH, Popham DL, Dworkin J (2008) A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell 135: 486-496.
99. Hussain H, Branny P, Allan E (2006) A eukaryotic-type serine/threonine protein kinase is required for biofilm formation, genetic competence, and acid resistance in Streptococcus mutans. J Bacteriol 188: 1628-1632.
100. Kristich CJ, Wells CL, Dunny GM (2007) A eukaryotic-type Ser/Thr kinase in Enterococcus faecalis mediates antimicrobial resistance and intestinal persistence. Proc Natl Acad Sci U S A 104: 3508-3513.

101. Madec E, Laszkiewicz A, Iwanicki A, Obuchowski M, Seror S (2002) Characterization of a membrane-linked Ser/Thr protein kinase in Bacillus subtilis, implicated in developmental processes. Mol Microbiol 46: 571-586.
102. Liebeke M, Meyer H, Donat S, Ohlsen K, Lalk M (2010) A metabolomic view of Staphylococcus aureus and its ser/thr kinase and phosphatase deletion mutants: involvement in cell wall biosynthesis. Chem Biol 17: 820-830.
103. Donat S, Streker K, Schirmeister T, Rakette S, Stehle T, et al. (2009) Transcriptome and functional analysis of the eukaryotic-type serine/threonine kinase PknB in Staphylococcus aureus. J Bacteriol 191: 4056-4069.
104. Lomas-Lopez R, Paracuellos P, Riberty M, Cozzone AJ, Duclos B (2007) Several enzymes of the central metabolism are phosphorylated in Staphylococcus aureus. FEMS Microbiol Lett 272: 35-42.
105. Tamber S, Schwartzman J, Cheung AL Role of PknB kinase in antibiotic resistance and virulence in community-acquired methicillin-resistant Staphylococcus aureus strain USA300. Infect Immun 78: 3637-3646.
106. Debarbouille M, Dramsi S, Dussurget O, Nahori MA, Vaganay E, et al. (2009) Characterization of a Serine/Threonine Kinase Involved in Virulence of Staphylococcus aureus. J Bacteriol.
107. Barton GM, Medzhitov R (2002) Toll-like receptors and their ligands. Curr Top Microbiol Immunol 270: 81-92.
108. Aderem A, Underhill DM (1999) Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 17: 593-623.
109. Grosdent N, Maridonneau-Parini I, Sory MP, Cornelis GR (2002) Role of Yops and adhesins in resistance of Yersinia enterocolitica to phagocytosis. Infect Immun 70: 4165-4176.
110. Welin A, Winberg ME, Abdalla H, Sarndahl E, Rasmusson B, et al. (2008) Incorporation of Mycobacterium tuberculosis lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. Infect Immun 76: 2882-2887.
111. Wei Z, Schnupf P, Poussin MA, Zenewicz LA, Shen H, et al. (2005) Characterization of Listeria monocytogenes expressing anthrolysin O and phosphatidylinositol-specific phospholipase C from Bacillus anthracis. Infect Immun 73: 6639-6646.
112. Pistor S, Chakraborty T, Niebuhr K, Domann E, Wehland J (1994) The ActA protein of Listeria monocytogenes acts as a nucleator inducing reorganization of the actin cytoskeleton. EMBO J 13: 758-763.
113. Tilney LG, Harb OS, Connelly PS, Robinson CG, Roy CR (2001) How the parasitic bacterium Legionella pneumophila modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J Cell Sci 114: 4637-4650.
114. Foster TJ, McDevitt D (1994) Surface-associated proteins of Staphylococcus aureus: their possible roles in virulence. FEMS Microbiol Lett 118: 199-205.
115. Lowy FD (1998) Staphylococcus aureus infections. N Engl J Med 339: 520-532.
116. Diks SH, Kok K, O'Toole T, Hommes DW, van Dijken P, et al. (2004) Kinome profiling for studying lipopolysaccharide signal transduction in human peripheral blood mononuclear cells. J Biol Chem 279: 49206-49213.
117. Miller M, Donat S, Rakette S, Stehle T, Kouwen TR, et al. (2010) Staphylococcal PknB as the first prokaryotic representative of the proline-directed kinases. PLoS One 5: e9057.
118. Taher TE, Parikh K, Flores-Borja F, Mletzko S, Isenberg DA, et al. Protein phosphorylation and kinome profiling reveal altered regulation of multiple signaling pathways in B lymphocytes from patients with systemic lupus erythematosus. Arthritis Rheum 62: 2412-2423.
119. Ritsema T, Peppelenbosch MP (2009) Kinome profiling of sugar signaling in plants using multiple platforms. Plant Signal Behav 4.
120. Della Bianca V, Grzeskowiak M, Rossi F (1990) Studies on molecular regulation of phagocytosis and activation of the NADPH oxidase in neutrophils. IgG- and C3b-mediated ingestion and associated respiratory burst independent of phospholipid turnover and Ca2+ transients. J Immunol 144: 1411-1417.
121. Myers JT, Swanson JA (2002) Calcium spikes in activated macrophages during Fcgamma receptor-mediated phagocytosis. J Leukoc Biol 72: 677-684.
122. Tan Z, Boss WF (1992) Association of Phosphatidylinositol Kinase, Phosphatidylinositol Monophosphate Kinase, and Diacylglycerol Kinase with the Cytoskeleton and F-Actin Fractions of Carrot (Daucus carota L.) Cells Grown in Suspension Culture : Response to Cell Wall-Degrading Enzymes. Plant Physiol 100: 2116-2120.
123. Yamamoto K, Pardee JD, Reidler J, Stryer L, Spudich JA (1982) Mechanism of interaction of Dictyostelium severin with actin filaments. J Cell Biol 95: 711-719.
124. Zhu Q, Liu T, Clarke M (1993) Calmodulin and the contractile vacuole complex in mitotic cells of Dictyostelium discoideum. J Cell Sci 104 (Pt 4): 1119-1127.
125. Doring V, Veretout F, Albrecht R, Muhlbauer B, Schlatterer C, et al. (1995) The in vivo role of annexin VII (synexin): characterization of an annexin VII-deficient Dictyostelium mutant indicates an involvement in Ca(2+)-regulated processes. J Cell Sci 108 (Pt 5): 2065-2076.
126. Vogel HJ (1994) The Merck Frosst Award Lecture 1994. Calmodulin: a versatile calcium mediator protein. Biochem Cell Biol 72: 357-376.
127. Peters C, Mayer A (1998) Ca2+/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. Nature 396: 575-580.
128. Park JB, Farnsworth CC, Glomset JA (1997) Ca2+/calmodulin causes Rab3A to dissociate from synaptic membranes. J Biol Chem 272: 20857-20865.
129. Tejle K, Magnusson KE, Rasmusson B (2002) Phagocytosis and phagosome maturation are regulated by calcium in J774 macrophages interacting with unopsonized prey. Biosci Rep 22: 529-540.
130. Colombo MI, Beron W, Stahl PD (1997) Calmodulin regulates endosome fusion. J Biol Chem 272: 7707-7712.
131. Braun AP, Schulman H (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu Rev Physiol 57: 417-445.
132. Yang E, Schulman H (1999) Structural examination of autoregulation of multifunctional calcium/calmodulin-dependent protein kinase II. J Biol Chem 274: 26199-26208.
133. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, et al. (2000) Protein kinase C isozymes and the regulation of diverse cell responses. Am J Physiol Lung Cell Mol Physiol 279: L429-438.

134. Allen LA, Aderem A (1996) Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. J Exp Med 184: 627-637.
135. Roubey RA, Ross GD, Merrill JT, Walton F, Reed W, et al. (1991) Staurosporine inhibits neutrophil phagocytosis but not iC3b binding mediated by CR3 (CD11b/CD18). J Immunol 146: 3557-3562.
136. Martin TF (1998) Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. Annu Rev Cell Dev Biol 14: 231-264.
137. Cox D, Tseng CC, Bjekic G, Greenberg S (1999) A requirement for phosphatidylinositol 3-kinase in pseudopod extension. J Biol Chem 274: 1240-1247.
138. Vieira OV, Bucci C, Harrison RE, Trimble WS, Lanzetti L, et al. (2003) Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. Mol Cell Biol 23: 2501-2514.
139. Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, et al. (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev 11: 701-713.
140. Vanhaesebroeck B, Alessi DR (2000) The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 346 Pt 3: 561-576.
141. Bonnema JD, Karnitz LM, Schoon RA, Abraham RT, Leibson PJ (1994) Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase C-independent granule release and cell-mediated cytotoxicity. J Exp Med 180: 1427-1435.
142. Dharmawardhane S, Sanders LC, Martin SS, Daniels RH, Bokoch GM (1997) Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. J Cell Biol 138: 1265-1278.
143. Sells MA, Chernoff J (1997) Emerging from the Pak: the p21-activated protein kinase family. Trends Cell Biol 7: 162-167.
144. Gurumurthy RK, Maurer AP, Machuy N, Hess S, Pleissner KP, et al. A loss-of-function screen reveals Ras- and Raf-independent MEK-ERK signaling during Chlamydia trachomatis infection. Sci Signal 3: ra21.
145. Goto H, Tanabe K, Manser E, Lim L, Yasui Y, et al. (2002) Phosphorylation and reorganization of vimentin by p21-activated kinase (PAK). Genes Cells 7: 91-97.
146. Tapinos N, Rambukkana A (2005) Insights into regulation of human Schwann cell proliferation by Erk1/2 via a MEK-independent and p56Lck-dependent pathway from leprosy bacilli. Proc Natl Acad Sci U S A 102: 9188-9193.
147. Koziel J, Maciag-Gudowska A, Mikolajczyk T, Bzowska M, Sturdevant DE, et al. (2009) Phagocytosis of Staphylococcus aureus by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors. PLoS ONE 4: e5210.
148. Majeed M, Caveggion E, Lowell CA, Berton G (2001) Role of Src kinases and Syk in Fcgamma receptor-mediated phagocytosis and phagosome-lysosome fusion. J Leukoc Biol 70: 801-811.
149. Elvin SJ, Williamson ED, Scott JC, Smith JN, Perez De Lema G, et al. (2004) Evolutionary genetics: Ambiguous role of CCR5 in Y. pestis infection. Nature 430: 417.
150. Snyderman R, Fudman EJ (1980) Demonstration of a chemotactic factor receptor on macrophages. J Immunol 124: 2754-2757.
151. Rahimpour R, Mitchell G, Khandaker MH, Kong C, Singh B, et al. (1999) Bacterial superantigens induce down-modulation of CC chemokine responsiveness in human monocytes via an alternative chemokine ligand-independent mechanism. J Immunol 162: 2299-2307.
152. Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, et al. (2003) Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. Proc Natl Acad Sci U S A 100: 13298-13302.
153. Reddy KB, Smith DM, Plow EF (2008) Analysis of Fyn function in hemostasis and alphaIIbbeta3-integrin signaling. J Cell Sci 121: 1641-1648.
154. Foster TJ, Hook M (1998) Surface protein adhesins of Staphylococcus aureus. Trends Microbiol 6: 484-488.
155. Fowler T, Wann ER, Joh D, Johansson S, Foster TJ, et al. (2000) Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur J Cell Biol 79: 672-679.
156. Shinji H, Seki K, Tajima A, Uchida A, Masuda S (2003) Fibronectin bound to the surface of Staphylococcus aureus induces association of very late antigen 5 and intracellular signaling factors with macrophage cytoskeleton. Infect Immun 71: 140-146.
157. Baranova IN, Kurlander R, Bocharov AV, Vishnyakova TG, Chen Z, et al. (2008) Role of human CD36 in bacterial recognition, phagocytosis, and pathogen-induced JNK-mediated signaling. J Immunol 181: 7147-7156.
158. Stuart LM, Deng J, Silver JM, Takahashi K, Tseng AA, et al. (2005) Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol 170: 477-485.
159. Watters DJ (2003) Oxidative stress in ataxia telangiectasia. Redox Rep 8: 23-29.
160. Hammond EM, Dorie MJ, Giaccia AJ (2003) ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. J Biol Chem 278: 12207-12213.
161. Wang X, Zalcenstein A, Oren M (2003) Nitric oxide promotes p53 nuclear retention and sensitizes neuroblastoma cells to apoptosis by ionizing radiation. Cell Death Differ 10: 468-476.
162. Hofseth LJ, Saito S, Hussain SP, Espey MG, Miranda KM, et al. (2003) Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. Proc Natl Acad Sci U S A 100: 143-148.
163. Watanabe I, Ichiki M, Shiratsuchi A, Nakanishi Y (2007) TLR2-mediated survival of Staphylococcus aureus in macrophages: a novel bacterial strategy against host innate immunity. J Immunol 178: 4917-4925.
164. Abe J, Okuda M, Huang Q, Yoshizumi M, Berk BC (2000) Reactive oxygen species activate p90 ribosomal S6 kinase via Fyn and Ras. J Biol Chem 275: 1739-1748.
165. Ibata-Ombetta S, Jouault T, Trinel PA, Poulain D (2001) Role of extracellular signal-regulated protein kinase cascade in macrophage killing of Candida albicans. J Leukoc Biol 70: 149-154.
166. Mitchell PJ, Barker KT, Martindale JE, Kamalati T, Lowe PN, et al. (1994) Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours. Oncogene 9: 2383-2390.
167. Kamalati T, Jolin HE, Fry MJ, Crompton MR (2000) Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation. Oncogene 19: 5471-5476.

168. Hong Q, Sze CI, Lin SR, Lee MH, He RY, et al. (2009) Complement C1q activates tumor suppressor WWOX to induce apoptosis in prostate cancer cells. PLoS One 4: e5755.
169. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, et al. (2002) Identification of a factor that links apoptotic cells to phagocytes. Nature 417: 182-187.
170. Eymann C, Homuth G, Scharf C, Hecker M (2002) Bacillus subtilis functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. J Bacteriol 184: 2500-2520.
171. Pane-Farre J, Jonas B, Forstner K, Engelmann S, Hecker M (2006) The sigmaB regulon in Staphylococcus aureus and its regulation. Int J Med Microbiol 296: 237-258.
172. Otto M, O'Mahoney DS, Guina T, Klebanoff SJ (2004) Activity of Staphylococcus epidermidis phenol-soluble modulin peptides expressed in Staphylococcus carnosus. J Infect Dis 190: 748-755.
173. Mehlin C, Headley CM, Klebanoff SJ (1999) An inflammatory polypeptide complex from Staphylococcus epidermidis: isolation and characterization. J Exp Med 189: 907-918.
174. Debarbouille M, Dramsi S, Dussurget O, Nahori MA, Vaganay E, et al. (2009) Characterization of a serine/threonine kinase involved in virulence of Staphylococcus aureus. J Bacteriol 191: 4070-4081.
175. Hirschhausen N, Schlesier T, Schmidt MA, Gotz F, Peters G, et al. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. Cell Microbiol 12: 1746-1764.
176. Ritossa F (1996) Discovery of the heat shock response. Cell Stress Chaperones 1: 97-98.
177. Vabulas RM, Wagner H, Schild H (2002) Heat shock proteins as ligands of toll-like receptors. Curr Top Microbiol Immunol 270: 169-184.
178. Panjwani NN, Popova L, Srivastava PK (2002) Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. J Immunol 168: 2997-3003.
179. Blachere NE, Li Z, Chandawarkar RY, Suto R, Jaikaria NS, et al. (1997) Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. J Exp Med 186: 1315-1322.
180. Kumaraguru U, Pack CD, Rouse BT (2003) Toll-like receptor ligand links innate and adaptive immune responses by the production of heat-shock proteins. J Leukoc Biol 73: 574-583.
181. Dziewanowska K, Carson AR, Patti JM, Deobald CF, Bayles KW, et al. (2000) Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells. Infect Immun 68: 6321-6328.
182. Jacobsen F, Baraniskin A, Mertens J, Mittler D, Mohammadi-Tabrisi A, et al. (2005) Activity of histone H1.2 in infected burn wounds. J Antimicrob Chemother 55: 735-741.
183. Noll TM, Desponds C, Belli SI, Glaser TA, Fasel NJ (1997) Histone H1 expression varies during the Leishmania major life cycle. Mol Biochem Parasitol 84: 215-227.
184. Solioz N, Blum-Tirouvanziam U, Jacquet R, Rafati S, Corradin G, et al. (1999) The protective capacities of histone H1 against experimental murine cutaneous leishmaniasis. Vaccine 18: 850-859.
185. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, et al. (2004) Neutrophil extracellular traps kill bacteria. Science 303: 1532-1535.
186. Chow OA, von Kockritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, et al. Statins enhance formation of phagocyte extracellular traps. Cell Host Microbe 8: 445-454.
187. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, et al. (2006) DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Curr Biol 16: 396-400.
188. Beiter K, Wartha F, Albiger B, Normark S, Zychlinsky A, et al. (2006) An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. Curr Biol 16: 401-407.
189. Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, et al. (2009) Extracellular histones are major mediators of death in sepsis. Nat Med 15: 1318-1321.
190. Boleij A, Schaeps RM, de Kleijn S, Hermans PW, Glaser P, et al. (2009) Surface-exposed histone-like protein a modulates adherence of Streptococcus gallolyticus to colon adenocarcinoma cells. Infect Immun 77: 5519-5527.
191. Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, et al. (2006) A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. Mol Microbiol 62: 667-679.
192. Strober W (2001) Trypan blue exclusion test of cell viability. Curr Protoc Immunol Appendix 3: Appendix 3B.
193. Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, et al. (1982) Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res 42: 1530-1536.
194. Kreegipuu A, Blom N, Brunak S (1999) PhosphoBase, a database of phosphorylation sites: release 2.0. Nucleic Acids Res 27: 237-239.
195. van Baal JW, Rygiel AM, Milano F, Anderson M, Bergman JJ, et al. (2008) Gene expression profile comparison of Barrett's esophagus epithelial cell cultures and biopsies. Dis Esophagus 21: 628-633.
196. Miller M, Donat S, Rakette S, Stehle T, Kouwen TR, et al. Staphylococcal PknB as the first prokaryotic representative of the proline-directed kinases. PLoS One 5: e9057.
197. Rosqvist R, Forsberg A, Wolf-Watz H (1991) Microinjection of the Yersinia YopE cytotoxin in mammalian cells induces actin microfilament disruption. Biochem Soc Trans 19: 1131-1132.
198. Rosqvist R, Magnusson KE, Wolf-Watz H (1994) Target cell contact triggers expression and polarized transfer of Yersinia YopE cytotoxin into mammalian cells. EMBO J 13: 964-972.
199. Galyov EE, Hakansson S, Forsberg A, Wolf-Watz H (1993) A secreted protein kinase of Yersinia pseudotuberculosis is an indispensable virulence determinant. Nature 361: 730-732.
200. Juris SJ, Rudolph AE, Huddler D, Orth K, Dixon JE (2000) A distinctive role for the Yersinia protein kinase: actin binding, kinase activation, and cytoskeleton disruption. Proc Natl Acad Sci U S A 97: 9431-9436.
201. Wiley DJ, Shrestha N, Yang J, Atis N, Dayton K, et al. (2009) The activities of the Yersinia protein kinase A (YpkA) and outer protein J (YopJ) virulence factors converge on an eIF2alpha kinase. J Biol Chem 284: 24744-24753.
202. Navarro L, Koller A, Nordfelth R, Wolf-Watz H, Taylor S, et al. (2007) Identification of a molecular target for the Yersinia protein kinase A. Mol Cell 26: 465-477.

203. Munoz-Dorado J, Inouye S, Inouye M (1993) Eukaryotic-like protein serine/threonine kinases in Myxococcus xanthus, a developmental bacterium exhibiting social behavior. J Cell Biochem 51: 29-33.
204. Perez J, Castaneda-Garcia A, Jenke-Kodama H, Muller R, Munoz-Dorado J (2008) Eukaryotic-like protein kinases in the prokaryotes and the myxobacterial kinome. Proc Natl Acad Sci U S A 105: 15950-15955.
205. Av-Gay Y, Everett M (2000) The eukaryotic-like Ser/Thr protein kinases of Mycobacterium tuberculosis. Trends Microbiol 8: 238-244.
206. Wang J, Li C, Yang H, Mushegian A, Jin S (1998) A novel serine/threonine protein kinase homologue of Pseudomonas aeruginosa is specifically inducible within the host infection site and is required for full virulence in neutropenic mice. J Bacteriol 180: 6764-6768.
207. Motley ST, Lory S (1999) Functional characterization of a serine/threonine protein kinase of Pseudomonas aeruginosa. Infect Immun 67: 5386-5394.
208. Pallova P, Hercik K, Saskova L, Novakova L, Branny P (2007) A eukaryotic-type serine/threonine protein kinase StkP of Streptococcus pneumoniae acts as a dimer in vivo. Biochem Biophys Res Commun 355: 526-530.
209. Echenique J, Kadioglu A, Romao S, Andrew PW, Trombe MC (2004) Protein serine/threonine kinase StkP positively controls virulence and competence in Streptococcus pneumoniae. Infect Immun 72: 2434-2437.
210. Novakova L, Saskova L, Pallova P, Janecek J, Novotna J, et al. (2005) Characterization of a eukaryotic type serine/threonine protein kinase and protein phosphatase of Streptococcus pneumoniae and identification of kinase substrates. FEBS J 272: 1243-1254.
211. Donat S, Streker K, Schirmeister T, Rakette S, Stehle T, et al. (2009) Transcriptome and functional analysis of the eukaryotic-type ser/thr kinase PknB in Staphylococcus aureus. J Bacteriol.
212. Melly MA, Thomison JB, Rogers DE (1960) Fate of staphylococci within human leukocytes. J Exp Med 112: 1121-1130.
213. Kapral FA (1965) The phagocytosis and intracellular fate of staphylococci. Ann N Y Acad Sci 128: 285-300.
214. Rogers DE, Tompsett R (1952) The survival of staphylococci within human leukocytes. J Exp Med 95: 209-230.
215. Baughn RE, Bonventre (1975) Nonspecific resistance to Listeria monocytogenes in mice infected and elicited with Staphylococcus aureus. Med Microbiol Immunol 161: 243-252.
216. Hebert A, Sayasith K, Senechal S, Dubreuil P, Lagace J (2000) Demonstration of intracellular Staphylococcus aureus in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. FEMS Microbiol Lett 193: 57-62.
217. Jakab GJ, Green GM (1976) Defect in intracellular killing of Staphylococcus aureus within alveolar macrophages in Sendai virus-infected murine lungs. J Clin Invest 57: 1533-1539.
218. Michailova L, Stoitsova S, Markova N, Kussovski V, Jordanova M, et al. (2000) Interaction of alveolar macrophages with Staphylococcus aureus and induction of microbial L-forms during infection in rats. Int J Med Microbiol 290: 259-267.
219. Elliott GR, Peterson PK, Verbrugh HA, Freiberg MR, Hoidal JR, et al. (1982) Influence of subinhibitory concentrations of penicillin, cephalothin, and clindamycin on Staphylococcus aureus growth in human phagocytic cells. Antimicrob Agents Chemother 22: 781-784.
220. Foster TJ (2005) Immune evasion by staphylococci. Nat Rev Microbiol 3: 948-958.
221. Sibbald MJ, Ziebandt AK, Engelmann S, Hecker M, de Jong A, et al. (2006) Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol Mol Biol Rev 70: 755-788.
222. Arbibe L, Mira JP, Teusch N, Kline L, Guha M, et al. (2000) Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nat Immunol 1: 533-540.
223. Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, et al. (2004) Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. Science 304: 1800-1804.
224. Park H, Teja K, O'Shea JJ, Siegel RM (2007) The Yersinia effector protein YpkA induces apoptosis independently of actin depolymerization. J Immunol 178: 6426-6434.
225. Wiley DJ, Nordfeldth R, Rosenzweig J, DaFonseca CJ, Gustin R, et al. (2006) The Ser/Thr kinase activity of the Yersinia protein kinase A (YpkA) is necessary for full virulence in the mouse, mollifying phagocytes, and disrupting the eukaryotic cytoskeleton. Microb Pathog 40: 234-243.
226. Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, van Dijl JM (2000) Signal peptide-dependent protein transport in Bacillus subtilis: a genome-based survey of the secretome. Microbiol Mol Biol Rev 64: 515-547.
227. Westers L, Westers H, Zanen G, Antelmann H, Hecker M, et al. (2008) Genetic or chemical protease inhibition causes significant changes in the Bacillus subtilis exoproteome. Proteomics 8: 2704-2713.
228. Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR, et al. (1999) Fibronectin binding protein and host cell tyrosine kinase are required for internalization of Staphylococcus aureus by epithelial cells. Infect Immun 67: 4673-4678.
229. Lammers A, Nuijten PJ, Smith HE (1999) The fibronectin binding proteins of Staphylococcus aureus are required for adhesion to and invasion of bovine mammary gland cells. FEMS Microbiol Lett 180: 103-109.
230. Ahmed S, Meghji S, Williams RJ, Henderson B, Brock JH, et al. (2001) Staphylococcus aureus fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. Infect Immun 69: 2872-2877.
231. Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA, et al. (1998) Intracellular Staphylococcus aureus escapes the endosome and induces apoptosis in epithelial cells. Infect Immun 66: 336-342.
232. Qazi SN, Counil E, Morrissey J, Rees CE, Cockayne A, et al. (2001) agr expression precedes escape of internalized Staphylococcus aureus from the host endosome. Infect Immun 69: 7074-7082.
233. Li XY, Green MR (1996) Intramolecular inhibition of activating transcription factor-2 function by its DNA-binding domain. Genes Dev 10: 517-527.
234. Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, et al. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 270: 7420-7426.
235. Gupta S, Campbell D, Derijard B, Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science 267: 389-393.

236. Livingstone C, Patel G, Jones N (1995) ATF-2 contains a phosphorylation-dependent transcriptional activation domain. EMBO J 14: 1785-1797.
237. Vlahopoulos SA, Logotheti S, Mikas D, Giarika A, Gorgoulis V, et al. (2008) The role of ATF-2 in oncogenesis. Bioessays 30: 314-327.
238. Lin YS, Green MR (1988) Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1a- and cyclic AMP-inducible promoters. Proc Natl Acad Sci U S A 85: 3396-3400.
239. Beier F, Taylor AC, LuValle P (2000) Activating transcription factor 2 is necessary for maximal activity and serum induction of the cyclin A promoter in chondrocytes. J Biol Chem 275: 12948-12953.
240. Laferriere J, Houle F, Taher MM, Valerie K, Huot J (2001) Transendothelial migration of colon carcinoma cells requires expression of E-selectin by endothelial cells and activation of stress-activated protein kinase-2 (SAPK2/p38) in the tumor cells. J Biol Chem 276: 33762-33772.
241. Maekawa T, Bernier F, Sato M, Nomura S, Singh M, et al. (1999) Mouse ATF-2 null mutants display features of a severe type of meconium aspiration syndrome. J Biol Chem 274: 17813-17819.
242. Ma Q, Li X, Vale-Cruz D, Brown ML, Beier F, et al. (2007) Activating transcription factor 2 controls Bcl-2 promoter activity in growth plate chondrocytes. J Cell Biochem 101: 477-487.
243. Song H, Ki SH, Kim SG, Moon A (2006) Activating transcription factor 2 mediates matrix metalloproteinase-2 transcriptional activation induced by p38 in breast epithelial cells. Cancer Res 66: 10487-10496.
244. Sevilla A, Santos CR, Vega FM, Lazo PA (2004) Human vaccinia-related kinase 1 (VRK1) activates the ATF2 transcriptional activity by novel phosphorylation on Thr-73 and Ser-62 and cooperates with JNK. J Biol Chem 279: 27458-27465.
245. Nezu J, Oku A, Jones MH, Shimane M (1997) Identification of two novel human putative serine/threonine kinases, VRK1 and VRK2, with structural similarity to vaccinia virus B1R kinase. Genomics 45: 327-331.
246. Vega FM, Gonzalo P, Gaspar ML, Lazo PA (2003) Expression of the VRK (vaccinia-related kinase) gene family of p53 regulators in murine hematopoietic development. FEBS Lett 544: 176-180.
247. Putcha GV, Le S, Frank S, Besirli CG, Clark K, et al. (2003) JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron 38: 899-914.
248. Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc Natl Acad Sci U S A 100: 2432-2437.
249. Puthalakath H, Strasser A (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ 9: 505-512.
250. Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, et al. (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 8: 705-711.
251. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 15: 1481-1486.
252. Black DS, Bliska JB (1997) Identification of p130Cas as a substrate of Yersinia YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. EMBO J 16: 2730-2744.
253. Haslinger-Loffler B, Kahl BC, Grundmeier M, Strangfeld K, Wagner B, et al. (2005) Multiple virulence factors are required for Staphylococcus aureus-induced apoptosis in endothelial cells. Cell Microbiol 7: 1087-1097.
254. Weglarczyk K, Baran J, Zembala M, Pryjma J (2004) Caspase-8 activation precedes alterations of mitochondrial membrane potential during monocyte apoptosis induced by phagocytosis and killing of Staphylococcus aureus. Infect Immun 72: 2590-2597.
255. Nilsdotter-Augustinsson A, Wilsson A, Larsson J, Stendahl O, Ohman L, et al. (2004) Staphylococcus aureus, but not Staphylococcus epidermidis, modulates the oxidative response and induces apoptosis in human neutrophils. APMIS 112: 109-118.
256. Barz C, Abahji TN, Trulzsch K, Heesemann J (2000) The Yersinia Ser/Thr protein kinase YpkA/YopO directly interacts with the small GTPases RhoA and Rac-1. FEBS Lett 482: 139-143.
257. Mills SD, Boland A, Sory MP, van der Smissen P, Kerbourch C, et al. (1997) Yersinia enterocolitica induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein. Proc Natl Acad Sci U S A 94: 12638-12643.
258. Nigg EA (2001) Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol 2: 21-32.
259. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, et al. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22: 153-183.
260. Lu KP, Liou YC, Vincent I (2003) Proline-directed phosphorylation and isomerization in mitotic regulation and in Alzheimer's Disease. Bioessays 25: 174-181.
261. Beltramini AM, Mukhopadhyay CD, Pancholi V (2009) Modulation of cell wall structure and antimicrobial susceptibility by a Staphylococcus aureus eukaryote-like serine/threonine kinase and phosphatase. Infect Immun 77: 1406-1416.
262. Donat S, Streker K, Schirmeister T, Rakette S, Stehle T, et al. (2009) Transcriptome and functional analysis of the eukaryotic-type ser/thr kinase PknB in *Staphylococcus aureus*. J Bacteriol.
263. Jiang L, He L, Fountoulakis M (2004) Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. J Chromatogr A 1023: 317-320.
264. Sambrook J, Fritsch EF, T M (1989) Molecular cloning: a laboratory manual New York: Cold Spring Harbor Laboratory Press. .
265. Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, et al. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305: 709-712.
266. Norrander J, Kempe T, Messing J (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26: 101-106.
267. Stearns-Kurosawa DJ, Osuchowski MF, Valentine C, Kurosawa S, Remick DG (2011) The pathogenesis of sepsis. Annu Rev Pathol 6: 19-48.

268. Miller M, Dreisbach A, Otto A, Becher D, Bernhardt J, et al. (2011) Mapping of interactions between human macrophages and Staphylococcus aureus reveals an involvement of MAP kinase signaling in the host defense. J Proteome Res 10: 4018-4032.
269. Schmidt U, Boucheron N, Unger B, Ellmeier W (2004) The role of Tec family kinases in myeloid cells. Int Arch Allergy Immunol 134: 65-78.
270. Satterthwaite AB, Witte ON (2000) The role of Bruton's tyrosine kinase in B-cell development and function: a genetic perspective. Immunol Rev 175: 120-127.
271. Agerer F, Lux S, Michel A, Rohde M, Ohlsen K, et al. (2005) Cellular invasion by Staphylococcus aureus reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. J Cell Sci 118: 2189-2200.
272. Hayashida A, Bartlett AH, Foster TJ, Park PW (2009) Staphylococcus aureus beta-toxin induces lung injury through syndecan-1. Am J Pathol 174: 509-518.
273. Tajima A, Iwase T, Shinji H, Seki K, Mizunoe Y (2009) Inhibition of endothelial interleukin-8 production and neutrophil transmigration by Staphylococcus aureus beta-hemolysin. Infect Immun 77: 327-334.
274. O'Callaghan RJ, Callegan MC, Moreau JM, Green LC, Foster TJ, et al. (1997) Specific roles of alpha-toxin and beta-toxin during Staphylococcus aureus corneal infection. Infect Immun 65: 1571-1578.
275. Ira, Johnston LJ (2008) Sphingomyelinase generation of ceramide promotes clustering of nanoscale domains in supported bilayer membranes. Biochim Biophys Acta 1778: 185-197.
276. Esen M, Schreiner B, Jendrossek V, Lang F, Fassbender K, et al. (2001) Mechanisms of Staphylococcus aureus induced apoptosis of human endothelial cells. Apoptosis 6: 431-439.
277. Grassme H, Becker KA, Zhang Y, Gulbins E (2008) Ceramide in bacterial infections and cystic fibrosis. Biol Chem 389: 1371-1379.
278. Walev I, Weller U, Strauch S, Foster T, Bhakdi S (1996) Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of Staphylococcus aureus. Infect Immun 64: 2974-2979.
279. Verkaik NJ, Benard M, Boelens HA, de Vogel CP, Nouwen JL, et al. Immune evasion cluster-positive bacteriophages are highly prevalent among human Staphylococcus aureus strains, but they are not essential in the first stages of nasal colonization. Clin Microbiol Infect 17: 343-348.
280. Ruggiero A, Squeglia F, Izzo V, Silipo A, Vitagliano L, et al. Expression, purification, crystallization and preliminary X-ray crystallographic analysis of the peptidoglycan binding region of the Ser/Thr kinase PrkC from Staphylococcus aureus. Protein Pept Lett 17: 1296-1299.
281. Maestro B, Novakova L, Hesek D, Lee M, Leyva E, et al. Recognition of peptidoglycan and beta-lactam antibiotics by the extracellular domain of the Ser/Thr protein kinase StkP from Streptococcus pneumoniae. FEBS Lett 585: 357-363.
282. Paracuellos P, Ballandras A, Robert X, Kahn R, Herve M, et al. The extended conformation of the 2.9-A crystal structure of the three-PASTA domain of a Ser/Thr kinase from the human pathogen Staphylococcus aureus. J Mol Biol 404: 847-858.
283. De Kimpe SJ, Kengatharan M, Thiemermann C, Vane JR (1995) The cell wall components peptidoglycan and lipoteichoic acid from Staphylococcus aureus act in synergy to cause shock and multiple organ failure. Proc Natl Acad Sci U S A 92: 10359-10363.
284. Middelveld RJ, Alving K (2000) Synergistic septicemic action of the gram-positive bacterial cell wall components peptidoglycan and lipoteichoic acid in the pig in vivo. Shock 13: 297-306.
285. Shimada T, Park BG, Wolf AJ, Brikos C, Goodridge HS, et al. (2010) Staphylococcus aureus evades lysozyme-based peptidoglycan digestion that links phagocytosis, inflammasome activation, and IL-1beta secretion. Cell Host Microbe 7: 38-49.
286. Boiarinova LV, Salina EV, Maianskaia IV, Maianskii AN (1983) [Reactivity to Staphylococcus aureus peptidoglycan in a neutrophil phagocytosis system]. Zh Mikrobiol Epidemiol Immunobiol: 49-53.
287. Tabuchi Y, Shiratsuchi A, Kurokawa K, Gong JH, Sekimizu K, et al. Inhibitory role for D-alanylation of wall teichoic acid in activation of insect Toll pathway by peptidoglycan of Staphylococcus aureus. J Immunol 185: 2424-2431.
288. van de Veerdonk FL, Joosten LA, Devesa I, Mora-Montes HM, Kanneganti TD, et al. (2009) Bypassing pathogen-induced inflammasome activation for the regulation of interleukin-1beta production by the fungal pathogen Candida albicans. J Infect Dis 199: 1087-1096.
289. Schellevis MA, Stoffels M, Hoppenreijs EP, Bodar E, Simon A, et al. (2011) Variable expression and treatment of PAPA syndrome. Ann Rheum Dis 70: 1168-1170.
290. Ziebandt AK, Kusch H, Degner M, Jaglitz S, Sibbald MJ, et al. Proteomics uncovers extreme heterogeneity in the Staphylococcus aureus exoproteome due to genomic plasticity and variant gene regulation. Proteomics 10: 1634-1644.
291. Rooijakkers SH, van Kessel KP, van Strijp JA (2005) Staphylococcal innate immune evasion. Trends Microbiol 13: 596-601.
292. Dreisbach A, van Dijl JM, Buist G (2011) The cell surface proteome of Staphylococcus aureus. Proteomics 11: 3154-3168.
293. Sibbald MJ, Winter T, van der Kooi-Pol MM, Buist G, Tsompanidou E, et al. Synthetic effects of secG and secY2 mutations on exoproteome biogenesis in Staphylococcus aureus. J Bacteriol 192: 3788-3800.
294. Driessen AJ, Nouwen N (2008) Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem 77: 643-667.
295. Papanikou E, Karamanou S, Economou A (2007) Bacterial protein secretion through the translocase nanomachine. Nat Rev Microbiol 5: 839-851.
296. Yuan J, Zweers JC, van Dijl JM, Dalbey RE (2010) Protein transport across and into cell membranes in bacteria and archaea. Cell Mol Life Sci 67: 179-199.
297. Tjalsma H, Antelmann H, Jongbloed JD, Braun PG, Darmon E, et al. (2004) Proteomics of protein secretion by Bacillus subtilis: separating the "secrets" of the secretome. Microbiol Mol Biol Rev 68: 207-233.
298. Kontinen VP, Sarvas M (1993) The PrsA lipoprotein is essential for protein secretion in Bacillus subtilis and sets a limit for high-level secretion. Mol Microbiol 8: 727-737.

299. Williams RC, Rees ML, Jacobs MF, Pragai Z, Thwaite JE, et al. (2003) Production of Bacillus anthracis protective antigen is dependent on the extracellular chaperone, PrsA. J Biol Chem 278: 18056-18062.
300. Kouwen TR, van Dijl JM (2009) Interchangeable modules in bacterial thiol-disulfide exchange pathways. Trends Microbiol 17: 6-12.
301. Kouwen TR, van der Goot A, Dorenbos R, Winter T, Antelmann H, et al. (2007) Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. Mol Microbiol 64: 984-999.
302. Heras B, Kurz M, Jarrott R, Shouldice SR, Frei P, et al. (2008) Staphylococcus aureus DsbA does not have a destabilizing disulfide. A new paradigm for bacterial oxidative folding. J Biol Chem 283: 4261-4271.
303. Lasica AM, Jagusztyn-Krynicka EK (2007) The role of Dsb proteins of Gram-negative bacteria in the process of pathogenesis. FEMS Microbiol Rev 31: 626-636.
304. Heras B, Shouldice SR, Totsika M, Scanlon MJ, Schembri MA, et al. (2009) DSB proteins and bacterial pathogenicity. Nat Rev Microbiol 7: 215-225.
305. Dutton RJ, Boyd D, Berkmen M, Beckwith J (2008) Bacterial species exhibit diversity in their mechanisms and capacity for protein disulfide bond formation. Proc Natl Acad Sci U S A 105: 11933-11938.
306. Barbieri JT, Sun J (2004) Pseudomonas aeruginosa ExoS and ExoT. Rev Physiol Biochem Pharmacol 152: 79-92.
307. Godlewska R, Dzwonek A, Mikula M, Ostrowski J, Pawlowski M, et al. (2006) Helicobacter pylori protein oxidation influences the colonization process. Int J Med Microbiol 296: 321-324.
308. Miki T, Okada N, Danbara H (2004) Two periplasmic disulfide oxidoreductases, DsbA and SrgA, target outer membrane protein SpiA, a component of the Salmonella pathogenicity island 2 type III secretion system. J Biol Chem 279: 34631-34642.
309. Peek JA, Taylor RK (1992) Characterization of a periplasmic thiol:disulfide interchange protein required for the functional maturation of secreted virulence factors of Vibrio cholerae. Proc Natl Acad Sci U S A 89: 6210-6214.
310. Cristobal S, de Gier JW, Nielsen H, von Heijne G (1999) Competition between Sec- and TAT-dependent protein translocation in Escherichia coli. EMBO J 18: 2982-2990.
311. Jongbloed JD, Martin U, Antelmann H, Hecker M, Tjalsma H, et al. (2000) TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway. J Biol Chem 275: 41350-41357.
312. Dilks K, Rose RW, Hartmann E, Pohlschroder M (2003) Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey. J Bacteriol 185: 1478-1483.
313. Berks BC, Palmer T, Sargent F (2005) Protein targeting by the bacterial twin-arginine translocation (Tat) pathway. Curr Opin Microbiol 8: 174-181.
314. Robinson C, Bolhuis A (2001) Protein targeting by the twin-arginine translocation pathway. Nat Rev Mol Cell Biol 2: 350-356.
315. Yamada K, Sanzen I, Ohkura T, Okamoto A, Torii K, et al. (2007) Analysis of twin-arginine translocation pathway homologue in Staphylococcus aureus. Curr Microbiol 55: 14-19.
316. Schmaler M, Jann NJ, Ferracin F, Landolt LZ, Biswas L, et al. (2009) Lipoproteins in Staphylococcus aureus mediate inflammation by TLR2 and iron-dependent growth in vivo. J Immunol 182: 7110-7118.
317. Morikawa K, Inose Y, Okamura H, Maruyama A, Hayashi H, et al. (2003) A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. Genes Cells 8: 699-712.
318. von Heijne G (1989) The structure of signal peptides from bacterial lipoproteins. Protein Eng 2: 531-534.
319. Kang HJ, Ha JM, Kim HS, Lee H, Kurokawa K, et al. (2011) The role of phagocytosis in IL-8 production by human monocytes in response to lipoproteins on Staphylococcus aureus. Biochem Biophys Res Commun 406: 449-453.
320. Dramsi S, Trieu-Cuot P, Bierne H (2005) Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. Res Microbiol 156: 289-297.
321. Ton-That H, Marraffini LA, Schneewind O (2004) Sortases and pilin elements involved in pilus assembly of Corynebacterium diphtheriae. Mol Microbiol 53: 251-261.
322. Ton-That H, Marraffini LA, Schneewind O (2004) Protein sorting to the cell wall envelope of Gram-positive bacteria. Biochim Biophys Acta 1694: 269-278.
323. Mazmanian SK, Ton-That H, Schneewind O (2001) Sortase-catalysed anchoring of surface proteins to the cell wall of Staphylococcus aureus. Mol Microbiol 40: 1049-1057.
324. Navarre WW, Schneewind O (1994) Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol Microbiol 14: 115-121.
325. Corrigan RM, Rigby D, Handley P, Foster TJ (2007) The role of Staphylococcus aureus surface protein SasG in adherence and biofilm formation. Microbiology 153: 2435-2446.
326. Josefsson E, Hartford O, O'Brien L, Patti JM, Foster T (2001) Protection against experimental Staphylococcus aureus arthritis by vaccination with clumping factor A, a novel virulence determinant. J Infect Dis 184: 1572-1580.
327. Mazmanian SK, Skaar EP, Gaspar AH, Humayun M, Gornicki P, et al. (2003) Passage of heme-iron across the envelope of Staphylococcus aureus. Science 299: 906-909.
328. Mazmanian SK, Ton-That H, Su K, Schneewind O (2002) An iron-regulated sortase anchors a class of surface protein during Staphylococcus aureus pathogenesis. Proc Natl Acad Sci U S A 99: 2293-2298.
329. Bentley ML, Gaweska H, Kielec JM, McCafferty DG (2007) Engineering the substrate specificity of Staphylococcus aureus Sortase A. The beta6/beta7 loop from SrtB confers NPQTN recognition to SrtA. J Biol Chem 282: 6571-6581.
330. Melvin JA, Murphy CF, Dubois LG, Thompson JW, Moseley MA, et al. (2011) Staphylococcus aureus sortase A contributes to the Trojan horse mechanism of immune defense evasion with its intrinsic resistance to Cys184 oxidation. Biochemistry 50: 7591-7599.
331. Charpentier X, Oswald E (2004) Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic Escherichia coli effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. J Bacteriol 186: 5486-5495.
332. Zlokarnik G, Negulescu PA, Knapp TE, Mere L, Burres N, et al. (1998) Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter. Science 279: 84-88.

333. Tsompanidou E, Sibbald MJ, Chlebowicz MA, Dreisbach A, Back JW, et al. (2011) Requirement of the agr locus for colony spreading of Staphylococcus aureus. J Bacteriol 193: 1267-1272.
334. Antelmann H, Tjalsma H, Voigt B, Ohlmeier S, Bron S, et al. (2001) A proteomic view on genome-based signal peptide predictions. Genome Res 11: 1484-1502.
335. Wang X, Xu M, Cai Y, Yang H, Zhang H, et al. (2009) Functional analysis of the disulphide loop mutant of staphylococcal enterotoxin C2. Appl Microbiol Biotechnol 82: 861-871.
336. Hovde CJ, Marr JC, Hoffmann ML, Hackett SP, Chi YI, et al. (1994) Investigation of the role of the disulphide bond in the activity and structure of staphylococcal enterotoxin C1. Mol Microbiol 13: 897-909.
337. Arriaga YL, Harville BA, Dreyfus LA (1995) Contribution of individual disulfide bonds to biological action of Escherichia coli heat-stable enterotoxin B. Infect Immun 63: 4715-4720.
338. Garred O, Dubinina E, Polesskaya A, Olsnes S, Kozlov J, et al. (1997) Role of the disulfide bond in Shiga toxin A-chain for toxin entry into cells. J Biol Chem 272: 11414-11419.
339. Kouwen TR, Antelmann H, van der Ploeg R, Denham EL, Hecker M, et al. (2009) MscL of Bacillus subtilis prevents selective release of cytoplasmic proteins in a hypotonic environment. Proteomics 9: 1033-1043.
340. Kouwen TR, Trip EN, Denham EL, Sibbald MJ, Dubois JY, et al. (2009) The large mechanosensitive channel MscL determines bacterial susceptibility to the bacteriocin sublancin 168. Antimicrob Agents Chemother 53: 4702-4711.
341. Kim HK, DeDent A, Cheng AG, McAdow M, Bagnoli F, et al. (2010) IsdA and IsdB antibodies protect mice against Staphylococcus aureus abscess formation and lethal challenge. Vaccine 28: 6382-6392.
342. Kim B, Richards SM, Gunn JS, Slauch JM (2010) Protecting against antimicrobial effectors in the phagosome allows SodCII to contribute to virulence in Salmonella enterica serovar Typhimurium. J Bacteriol 192: 2140-2149.
343. Hanada M, Nishiyama K, Tokuda H (1996) SecG plays a critical role in protein translocation in the absence of the proton motive force as well as at low temperature. FEBS Lett 381: 25-28.
344. Veenendaal AK, van der Does C, Driessen AJ (2004) The protein-conducting channel SecYEG. Biochim Biophys Acta 1694: 81-95.
345. Chaudhuri RR, Allen AG, Owen PJ, Shalom G, Stone K, et al. (2009) Comprehensive identification of essential Staphylococcus aureus genes using Transposon-Mediated Differential Hybridisation (TMDH). BMC Genomics 10: 291.
346. Forsgren A, Sjoquist J (1966) "Protein A" from *S. aureus*. I. Pseudo-immune reaction with human gamma-globulin. J Immunol 97: 822-827.
347. Gemmell CG. Susceptibility to opsonophagocytosis of protein A,alpha-haemolysin and beta-toxin deficient mutants of *S. aureus* isolated by allele-replacement.Proceedings of
the VIth International Symposium on Staphylococci and Staphylococcal Infections; 1991; Stuttgart. New York: Gustav Fisher Verlag. pp. 273-277.
348. Peterson PK, Verhoef J, Sabath LD, Quie PG (1977) Effect of protein A on staphylococcal opsonization. Infect Immun 15: 760-764.
349. Singh VK, Carlos MR, Singh K (2010) Physiological significance of the peptidoglycan hydrolase, LytM, in Staphylococcus aureus. FEMS Microbiol Lett 311: 167-175.
350. Lang S, Livesley MA, Lambert PA, Littler WA, Elliott TS (2000) Identification of a novel antigen from Staphylococcus epidermidis. FEMS Immunol Med Microbiol 29: 213-220.
351. Mackey-Lawrence NM, Potter DE, Cerca N, Jefferson KK (2009) Staphylococcus aureus immunodominant surface antigen B is a cell-surface associated nucleic acid binding protein. BMC Microbiol 9: 61.
352. Blair JE, Williams RE (1961) Phage typing of staphylococci. Bull World Health Organ 24: 771-784.
353. Lindsay JA (2008) Genetic manipulation of Staphylococcus aureus. Staphylococcus: molecular genetics Norfolk: Caister Academic Press
pp. 112.
354. Arnaud M, Chastanet A, Debarbouille M (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ Microbiol 70: 6887-6891.
355. Sibbald MJ (2009) The Staphylococcus aureus secretome. Groningen: Rijksuniversiteit Groningen.
356. de Jong MF, Sun YH, den Hartigh AB, van Dijl JM, Tsolis RM (2008) Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the Brucella type IV secretion system. Mol Microbiol 70: 1378-1396.
357. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, et al. (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. J Bacteriol 184: 5457-5467.

Chapter 10

Acknowledgments / Podziękowania

Acknowledgments

Working on the Ph.D. has been a wonderful and often overwhelming experience. But none said it would be easy ☺. It is hard for me to say whether it has been grappling with the topic itself which has been the real learning experience, or grappling with how to do experiments, write papers, give talks, be awake until the birds start singing, and stay focus… But I am happy that I have accepted this challenge and even happier that I have won it ! I would have not been able to finish this grappling alone, since this "success has many fathers". Acknowledging many people who have helped me to achieve this doctorate degree has turned out to be the most challenging part of this dissertation. This is because, I have received countless support from dozens of people and thanking every individual without missing anybody is a difficult task.

First, I would like to thank my promoter, Prof. dr. Jan Maarten van Dijl whose support and never-ending optimism showed to be vital for the finishing of this thesis. Dear Jan Maarten, thank you very much for giving me a chance to stay in your group after my Master degree and making this five years an incredible experience. I remember that in all my moments of weakness (and I had a lot of them) you always said "You will get there!" It was hard to believe then but I made it! And it would not be possible without your limitless support, especially during these long hours spent in your office while finishing our manuscripts. You have oriented and supported me with promptness and care, and you have always been patient and encouraging and you were open to my ideas during discussions, which frequently led to key insights. I hope that we will have many chances to collaborate in the future.

Next I would like to thank my second promoter, Prof. dr. Maikel P. Peppelenbosch. Dear Maikel, I am very glad that you were my promoter and I am very grateful for insightful comments concerning both my work and this thesis. Thank you for your support, and for many motivating discussions. I really appreciate your interest in learning foreign languages, especially I am impressed with your knowledge of Polish language, which you have mastered during your internship in Poland: Drogi Maikelu, to była ogromna przyjemność pracować z Tobą podczas mojego doktoratu. Bardzo dziękuję Ci za Twoje wsparcie, pogodę ducha, cierpliwość, ciekawe dyskusje i błyskotliwe spojrzenie na świat ☺. Dziękuję Ci za litry oddanej krwi do eksperymentów (wciąż jestem Ci winna posiłek regeneracyjny ☺) i za pomoc w laboratorium podczas moich pierwszych, długich eksperymentów z monocytami. Ponadto podziwiam Twoją zdolność do objaśniania rzeczy skomplikowanych w prosty sposób, co znacznie ułatwiło mi analizę wyników. Dziękuję za wszystko!

I would like to thank my co-promoter, Dr. Annette Dreisbach. Dear Annette, the great officemate, colleague, supervisor and friend, thank you very much for all these years we spend together in the same office, for a great atmosphere and for showing me VLOOKUP function in Excel ☺. And most importantly I am grateful for your constructive criticism, daily supervision and help with experiments.

Dear reading committee of my thesis, Prof. Dr. Tom van der Poll, Prof. Dr. Arie Jan van Winkelhoff and Prof. Dr. Uwe Völker, thank you for the critical reading and evaluation of my thesis.

I would especially like to thank Sander and Kaushal, for teaching me everything I know about pepchips and helping me with the kinome profiling data analysis. It was a pleasure to meet you and I wish you all the best in your future career.

A big THANK YOU to all my colleagues (past and present) from MolBac; Thijs, René, Marc, Jean-Yves, Monika, Sjouke, Jessica, Emma, Marloes, Eleni, Corrina, Ewoud, Lakshmi, Carmine, Henrik, Vahid, Federico, Jolanda, Rense, Denis, Artur, Vivianne, Marcus, Ruben, Jetta, Girbe and Sierd for your help with various things and making the lab of MolBac pleasant environment to work in.

Many thanks to people from the Department of Medical Microbiology and Immunology at UC Davis, especially to Prof. Dr. Renee Tsolis and Prof. Dr. Andreas Bäumler. Dear Renee, thank you very much for giving me the opportunity to work in your laboratory. Maarten, thank for your help with penicillinase translocation assay, confocal microscopy and great time I had during my stay in Davis. Mari, Maria, Marijke, Kim, Torsten, Paul, Sebastian, Parameth, Sean-Paul, Alanna and other colleagues from Renee and Andreas lab - thank you all for unforgettable moments!

I would like to thank many collaborators in the field with whom I have worked together on different projects. Without these fruitful collaborations, none of the chapters of this thesis would have been possible. Many thanks to Andreas Otto, Dörte Becher, Jörg Bernhardt, Katrin Gronau and Michael Hecker from Greifswald for the collaboration we had on PknB phosphorylation properties and on interactions between THP-1 macrophages and *S. aureus.* Thank you Knut, Stefanie, Sonja and Thilo for purified PknB and *pknB* mutant strains and the joint paper we wrote. Thank you Jiri and Lucie for a great collaboration on penicillinase translocation project. You helped me a lot with strains and phage transduction. I it a pity that the project did not give publishable results, but who knows what future might bring.

Thank you to all the people from my current lab in Nijmegen (Experimental Internal Medicine) for the motivating and great working environment. Good luck with all your projects and your future career. Dear Leo and Mihai, thank you very much for giving me the chance to work with you and for your faith and confidence that I will finish this thesis.

To my invaluable paranymphs, Magda and Agnieszka both of you have provided a lot of support and help with preparations for my graduation. Magda, dziękuję Tobie i Heniowi za pomoc i cierpliwość w moich niekończących się przeprowadzkach :) i te wszystkie godziny, które spędziłyśmy razem na pogaduchach. Agnieszka, dzięki wielkie za wszystko a w szczególności za przechowanie mnie u siebie, kiedy przyjeżdżałam do Groningen. Dziękuję za ten czas spędzony z Tobą i Deepakiem, wszystkie rozmowy z Wami zawsze podnosiły mnie na duchu i motywowały do działania. Dear Deepak, thanks a lot for your optimism, support and great sense of humor :)

Droga Olu - sąsiadko „z naprzeciwka", dziękuję Ci za wszystkie nasze rozmowy, szczególnie te balkonowe z butelką czerwonego wina i kostką świeżego parmezanu :) Strasznie mi tego brakuje...

Drogie Antki: Kasiu i Antku, dziękuję Wam za Wasze wielkie wsparcie, za czas spędzony na rozmowach, za poczucie humoru i Waszą przyjaźń .

Marysiu, dziękuję Ci za przygarnięcie mnie do siebie po przyjeździe do Nijmegen, za pyszne pierogi, gościnność, otwartość, zaufanie i przyjaźń.

Serdecznie podziękowania dla całej mojej rodziny: babci Reli, babci Heli, dziadka Tadeusza, wujka Tadeusza, cioci Wiesi, cioci Grażynki, wujka Heńka, wujka Mirka, Jarka, Kasi, Zosi, Antosia, Piotrka, Pawła, Anety, Michała, Jacka, Ani, mamy Władzi i taty Leszka, babci Janki, dziadka Władysława, babci Bronisławy, cioci Zosi, cioci Basi, wujka Cześka i Łukasza. Dziękuję Wam za Wasze ciepłe przyjęcie za każdym razem jak jesteśmy w Łodzi lub Spytkowicach.

Kochana Mamo, już od dwóch lat kończę ten doktorat i kończę... Ale teraz to już naprawdę koniec! Dziękuję za Twoje wsparcie, wyrozumiałość, cierpliwość i pogaduchy przez telefon, które zawsze dodawały mi siły i podnosiły na duchu. Dziękuję za Wszystko!

Kochany mężu :) Gdyby nie Ty, to chyba bym w życiu nie skończyła tego doktoratu! Dziękuję za Twoją niebiańską (do mnie) cierpliwość, za poczucie humoru, i za to, że po prostu zawsze przy mnie jesteś!

The printing of this thesis was financially supported by:

University of Groningen, Groningen, the Netherlands

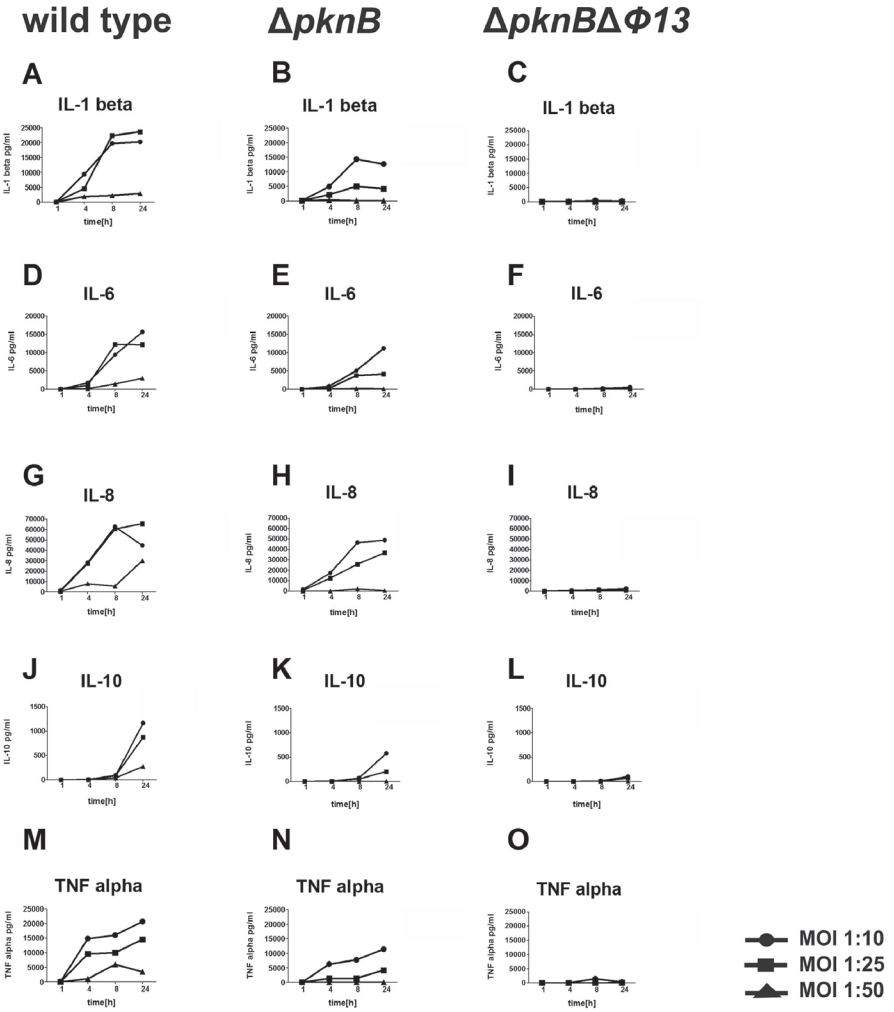
Research Institute for Drug Exploration, Groningen, the Netherland

Peptide products and services, discovery of new therapeutic drugs, Lelystad, the Netherlands

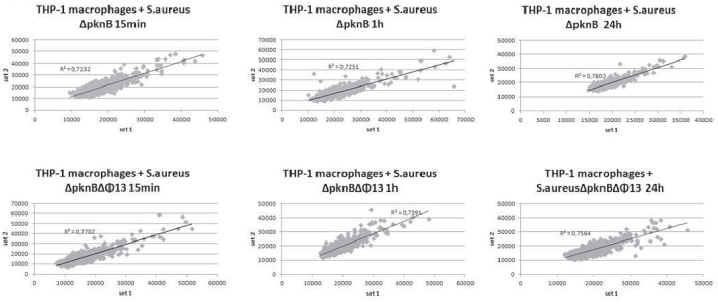
Chapter 11

Supplementary data

Supplemental Figure 1. Production of inflammatory mediators by PBMCs stimulated with *S. aureus* strains at different MOIs



Supplemental Figure 2. Correlation plots after comparison of different data sets for THP-1 macrophages incubated with different strains of *S. aureus.*



Supplemental Table 1. Kinome profiling of THP-1 macrophages incubated with *S. aureus* NCTC 8325 wild type

Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
51	TSLAQYDSNSK	BMX non-receptor tyrosine kinase Etk	Btk	0,25	1	0,64	tyrosine protein kinase	IL-6 induction, apoptosis, cell structure disassembly during apoptosis
84	LGSQSYEDMRG	CD19	Btk	0,72	1	0,83	cell surface	regulation of immune response; cell surface receptor linked signal transduction; cellular defense response
376	KVVALYDYMPM	Bruton's tyrosine kinase	Btk	0,38	1	1	tyrosine protein kinase	calcium-mediated signaling, induction of apoptosis by extracellular signals
567	NQLFLYDTHQN	Phospholipase C, gamma 2	Btk	0,75	1	1	phospholipase	elevation of cytosolic calcium ion concentration, negative regulation of programmed cell death,
52	SANAIYSLAAR	CBL	EGFR	0,7	1	0,88	adaptor protein	positive regulation of receptor-mediated endocytosis
277	ATLDVYNPFET	SCAMP3	EGFR	0,58	1,18	1	vesicle protein	protein transport, endocytosis, post-Golgi vesicle-mediated transport, response to chemical stimulus
348	LPVPEYINQSV	EGF receptor	EGFR	0,76	1	1	Receptor tyrosine kinase	cell-cell adhesion,negative regulation of apoptosis, positive regulation of nitric oxide biosynthetic process, positive regulation of protein kinase B signaling cascade, response to stress
533	PGLDEYNPFSD	SCAMP1	EGFR	0,47	1	1	vesicle protein	protein transport; post-Golgi vesicle-mediated transport
537	LKSPAYRDLAA	Regulator of G protein signaling 16	EGFR	0,76	1	1	GTPase activating pr	regulation of G-protein coupled receptor protein signaling pathway; negative regulation of signal transduction
604	LRLQDYEEKTK	Ezrin	EGFR	0,51	1	1	cytoskeletal protein	actin filament bundle formation, regulation of cell shape, leukocyte adhesion, cytoskeletal anchoring, membrane to membrane docking
793	AENPEYLGLDV	ErbB2	EGFR	0,74	1	1	receptor tyrosine kinase	phosphoinositide 3-kinase cascade, positive regulation of cell adhesio, positive regulation of MAPKKK cascade,positive regulation of Ras protein signal transduction
860	MFPRNYVTPVN	Grb2	EGFR	0,91	0,7	1	Adaptor/scaffold	DNA damage response, signal transduction, cell-cell signaling; regulation of MAPKKK cascade, receptor internalization, Ras protein signal transduction
92	DGKEIYNTIRR	RasGAP	EGFR;Lck	0,61	1	0,53	GTPase activating protein	regulation of cell shape, regulation of actin filament polymerization, regulation of small GTPase mediated signal transduction,negative regulation of cell adhesion,positive regulation of anti-apoptosis, negative regulation of cell-matrix adhesion
225	AGPALSPVPPV	Bcl 2	ERK	1,71	1	1	transcription factor	Autophagy, anti-apoptosis, humoral immune response, response to toxin,oxygen and reactive oxygen species metabolic process
248	SDSLSSPTLLA	c-Fos	ERK	0,34	1	1	transcription factor	response to cAMP, response to toxin, stress-activated MAPK cascade, inflammatory response, MyD88-independent toll-like receptor signaling pathway, innate immune response
378	LQGFNSPGMLS	Myocyte specific enhancer factor 2A	ERK	0,76	1	1	transcription factor	apoptosis, MyD88-independent toll-like receptor signaling pathway, stress-activated MAPK cascade, innate immune response
468	NLLPMSPEEFD	STAT1	ERK	0,53	1	0,74	transcription factor	response to bacterium, response to cytokine stimulus, induction of apoptosis
496	KVEPASPPYYS	Peroxisome proliferator activated receptor, gamma	ERK	0,46	1	1	transcription factor	induction of apoptosis, innate immune response, negative regulation of acute inflammatory response
504	LVSPDSPRSID	Leukemia inhibitory factor receptor, alpha	ERK	0,42	1	1	cytokine receptor	positive regulation of anti-apoptosis, response to cytokine stimulus
597	RTAPYTPNLPH	SMAD4	ERK	0,67	1	1	transcription factor	negative regulation of cell growth, response to hypoxia,

Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
629	LVEPLTPSGEA	EGF receptor	ERK	0,66	1	1	Receptor tyrosine kinase	response to oxidative stress, positive regulation of MAP kinase activity
685	RRRFSSLHFMV	Caspase 9	ERK	1,67	1	1	protease	induction of apoptosis, phosphoinositide-mediated signaling
1008	PTAPLSPMSPP	Connexin 43	ERK	0,67	1	1	Channel	positive regulation of I-kappaB kinase/NF-kappaB cascade, apoptosis
18	IESDIYAEIPD	Focal adhesion kinase	FAK	0,78	1	1	tyrosine protein kinase	integrin signaling, extracellular matrix organization and biogenesis, regulation of cell adhesion mediated by integrin
55	MEDYDYVHLQG	p130CAS	FAK	0,69	1	1	Adaptor/scaffold	integrin signaling, actin filament organization, G-protein coupled receptor protein signaling, positive regulation of cell migration
311	KTNLSYYEYDK	BMX non-receptor tyrosine kinase Etk	FAK	0,25	1	1	tyrosine protein kinase	IL-6 induction, apoptosis, cell structure disassembly during apoptosis
539	EEEHVYSFPNK	Paxillin	FAK	0,39	1	1	Adaptor/scaffold, Cytoskeletal protein, Adhesion	cell-matrix adhesion, cellular response to reactive oxygen species, cell junction assembly
795	SETDDYAEIID	Focal adhesion kinase	FAK	0,72	1	0,79	tyrosine protein kinase	integrin signaling, extracellular matrix organization and biogenesis, regulation of cell adhesion mediated by integrin
569	ADDSYYTARSA	ZAP70	Lck	0,57	1	1	tyrosine protein kinase	immune response,
825	EANSHYGHNDD	CD31	Lck;Csk	0,89	1	1	Adhesion	cell adhesion, leukocyte migration, phagocytosis
84	LGSQSYEDMRG	CD19	Lyn	0,72	1	0,83	cell surface	regulation of immune response, cellular defense response
557	ASKRSYQFWDT	N-Myristoyl transferase 1	Lyn	0,89	1	1	Transferase	activation of pro-apoptotic gene products
888	MRGILYAAPQL	CD19	Lyn	0,82	1	1	cell surface	regulation of immune response, cellular defense response
35	TATEGQYQQQP	Lyn	Src	2,15	1	0,71	tyrosine protein kinase	positive regulation of cell motility, positive regulation of tyrosine phosphorylation of STAT protein,
103	CGSQKYAYFNG	Connexin 43	Src	0,78	1	1	Channel	positive regulation of I-kappaB kinase/NF-kappaB cascade, gap junction assembly,
284	TEENIYQVPTS	DAB1	Src	0,71	1	1	Adaptor/scaffold	cell-cell adhesion, small GTPase mediated signal transduction,
332	EDSTYYKASKG	FAK	Src	0,58	1	0,83	tyrosine protein kinase	integrin-mediated signaling pathway, apoptosis, regulation of cell adhesion and phagocytosis
573	QQQEVYGMMPR	Spectrin	Src	0,81	1	1	cytoskeletal protein	apoptosis, actin filament capping, cell structure disassembly during apoptosis
592	TTSQLYDAVPI	PDK1	Src	0,76	1	1	ser/thr kinase	phosphoinositide-mediated signaling, activation of protein kinase B, actin cytoskeleton organization and biogenesis
780	TPSAAYLWVGT	Gelsolin	Src	1,37	1	1	actin binding protein	regulation of cell adhesion, actin filament severing, phosphoinositide-mediated signaling, apoptosis, vesicle-mediated transport, actin filament polymerization, cell structure disassembly during apoptosis
50	FEGFSYVNPQF	Protein kinase C alpha	SYK	1	1	0,69	ser/thr kinase	activation of phospholipase C activity,induction of apoptosis by extracellular signals,
54	PSEEGYQDYEP	Synuclein alpha	SYK	0,66	1	1	Adaptor/scaffold	anti-apoptosis, negative regulation of exocytosis, positive regulation of endocytosis
530	VSFNPYEPELA	SYK	SYK	0,67	1	1	tyrosine protein kinase	integrin-mediated signaling pathway, neutrophil chemotaxis,
786	ADENYYKAQTH	SYK	SYK	0,52	1	1	tyrosine protein kinase	integrin-mediated signaling pathway, neutrophil chemotaxis,

Supplemental Table 2. Kinome profiling of THP-1 macrophages incubated with *S. aureus* NCTC 8325 Δ*pknB*

Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
632	PEPGPYAQPSV	CRK	ABL,EGFR	0,38	1,72	1	Adaptor/scaffold	regulation of Rac protein signal transduction, activation of MAPKK activity, actin cytoskeleton organization and biogenesis
727	RPRNYSVGSRP	6-phosphofructo-2-kinase	AKT	0,52	1,91	1	Kinase (non-protein)	glycolysis
175	RSRHSSYPAGT	BCL2 antagonist of cell death	AKT	1	1,48	0,74	Apoptosis	phosphoinositide-mediated signaling, induction of apoptosis by extracellular signals,
916	IIRQPSEEEII	PEA15	Akt, CaMKII	0,41	1,95	1	Apoptosis	anti-apoptosis and autophagy
93	RPRSCTWPLQR	Forkhead box protein O3A	Akt1	0,62	1,76	0,8	Autophagy, transcription factor	phosphoinositide-mediated signaling, inhibition of apoptosis,response to DNA damage stimulus
593	VLRPETPRPVD	Caspase 9	AKT1	0,52	1	0,8	Protease, Apoptosis	inhibition of apoptosis, phosphoinositide-mediated signaling, caspase activation via cytochrome c
173	RRRAVSMDNSN	Forkhead box protein O3A	AKT1	3,45	1	0,81	Transcription factor, DNA binding protein, Autophagy	induction of apoptosis, DNA damage response,
693	IRSSMSGLHLV	Acetyl-CoA carboxylase alpha	AMPK	0,44	1,7	1	ligase	Lipid Metabolism - fatty acid biosynthesis
354	EKCSDSQSWED	Tumor protein p53 binding protein 1	ATM	0,54	1,95	1	Transcription, coactivator/corepressor	DNA-dependent, DNA repair, double-strand break repair
21	CSGLSSQSDIL	BRCA1	ATM	0,31	1,52	1	Ubiquitin conjugating system, Transcription, Tumor suppressor	apoptosis, positive regulation of transcription, DNA damage response,
512	PIDMESQERIK	c-Jun	c-Abl, JNK	0,43	1,58	1	transcription factor	response to cAMP,response to cytokine stimulus, innate immune response, MyD88-independent toll-like receptor signaling pathway
249	LARRPSYRKIL	ATF-1	CaMKI, PKA, S6K	0,37	1,9	1	transcription factor	MyD88-independent toll-like receptor signaling pathway, stress-activated MAPK cascade, innate immune response
579	SSPPGTPSPAD	CCAAT	CaMKII	0,62	1	0,8	DNA binding protein, Transcription factor	regulation of interleukin-6 biosynthetic process,induction of apoptosis, acute-phase response, anti-apoptosis
409	PQPPKSPGPHS	RAD9	CDK1	0,4	1	0,72	Cell cycle regulation	positive regulation of apoptosis, DNA damage checkpoint, DNA replication
57	AQDEFYRSGWA	Csk	Csk	0,49	1,66	0,77	tyrosine kinase	epidermal growth factor receptor signaling pathway
128	PATDLYQVPPG	p130CAS	c-Src	0,47	1,48	0,78	Adaptor/scaffold	integrin-mediated signaling pathway, G-protein coupled receptor protein signaling pathway, actin filament organization, regulation of apoptosis
417	LSPLPSQAMDD	p53	DNAPK	0,75	1,77	1	transcription factor	
25	PELARYLNRNY	Hrs	EGFR	0,61	1,95	1	Adaptor/scaffold	negative regulation of JAK-STAT cascade
604	LRLQDYEEKTK	Ezrin	EGFR	0,28	1,39	1	Cytoskeletal protein	actin filament, actin cytoskeleton, filopodium
59	GGDDIYEDIIK	VAV2	EGFR	0,51	1,67	0,75	Guanine nucleotide exchange factor, Rac/Rho	lamellipodium biogenesis, positive regulation of phosphoinositide 3-kinase activity, small GTPase mediated signal transduction, regulation of Rho protein signal transduction
92	DGKEIYNTIRR	RasGAP	EGFR,Lck	0,39	1,42	0,75	GTPase activating protein	regulation of actin filament polymerization, regulation of small GTPase mediated signal transduction, positive regulation of anti-apoptosis

Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
629	LVEPLTPSGEA	EGF receptor	ERK1, ERK2	0,39	1,39	1	Receptor tyrosine kinase	positive regulation of nitric oxide biosynthetic process, activation of MAPKK activity, response to oxidative stress, negative regulation of apoptosis, positive regulation of cell migration
193	YVSILSPKEVS	Regulator of G protein signaling 19	ERK2	0,69	1,9	1	GTPase activating protein	G-protein coupled receptor protein signaling pathway, small GTPase mediated signal transduction, autophagy, negative regulation of signal transduction
853	IEQWFTEDPGP	p53	ERK2	0,4	1,84	1	transcription factor	
600	GVDGDYEDAEL	BCR	FES_kinase	0,53	1,74	1	Ser/Thr kinase,GTPase activating protein	regulation of small GTPase mediated signal transduction,
34	HHIDYYKKTTN	FGF receptor 1	FGFR1	0,69	1,97	1	Receptor tyrosine kinase	MAPKKK cascade, negative regulation of apoptosis
3	TTNEEYLDLSQ	Fibroblast growth factor receptor 2	FGFR2	0,72	2,23	1	Receptor tyrosine kinase	MAPKKK cascade, negative regulation of apoptosis
58	IKDDEYNPCQG	Fgr	Fgr	0,6	1,83	0,75	tyrosine kinase	innate immunity, defense response to Gram-positive bacterium, Functions as negative regulator of phagocytosis and SYK activity in monocytes
28	PCTTIYVAATE	SLAM	Fyn	0,45	1,62	0,78	Receptor	Host-bacteria interaction,sensor of bacteria
951	APERASSVYTR	CCR5	GRK	0,56	1,88	1	Receptor, GPCR	cellular defense response, entry into host cell, immune response, chemotaxis, inflammatory response
187	DDLMLSPDDIE	p53	nd	0,45	1,63	0,81	transcription factor	DNA damage response,induction of apoptosis by intracellular signals,
571	RGDKGYVPSVF	STAT4	IL-2, MAP2K6, STAT4	0,39	1,54	1	transcription factor	cytokine and chemokine mediated signaling pathway, JAK-STAT cascade
764	NTGEQSGYHVE	Beta-2-adrenergic receptor	Insulin receptor	0,53	1,63	1	Receptor, GPCR	G-protein signaling, adenylate cyclase activating pathway,positive regulation of MAPKKK cascade,endosome to lysosome transport, receptor-mediated endocytosis
53	LTIDRYLAIVH	CCR2	JAK2	0,46	1,61	1	Receptor, GPCR	cytokine and chemokine mediated signaling pathway, immune response, inflammatory response, cell adhesion, chemotaxis
29	GVITTTPTPPG	Oncogene Jun-B	JNK	0,66	1,71	0,76	transcription factor	response to cAMP,
338	EEIYLTPVQRP	MAPK8 interacting protein 1	JNK1	0,39	1	0,78	Adaptor/scaffold	vesicle-mediated transport, negative regulation of JNK cascade, negative regulation of JNK activity, regulation of transcription, DNA-dependent, anti-apoptosis, signal transduction, regulation of JNK cascade, JUN phosphorylation
225	AGPALSPVPPV	Bcl 2	JNK1, TNF-alpha	0,81	1	0,79	Membrane protein	focal adhesion formation,oxygen and reactive oxygen species metabolic process, anti-apoptosis
26	INGNNYVYIDP	KIT	KIT	0,52	1,77	0,77	receptor tyrosine kinase	negative regulation of programmed cell death, positive regulation of pseudopodium formation, positive regulation of MAP kinase activity
569	ADDSYYTARSA	ZAP70	Lck	0,36	1,55	1	tyrosine kinase	immune response,positive regulation of calcium-mediated signaling
825	EANSHYGHNDD	PECAM-1	Lck,Csk	0,49	1,69	1	Adhesion, Membrane protein	phagocytosis

Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
84	LGSQSYEDMRG	CD19	Lyn, Btk	0,44	1,45	1	Membrane protein	regulation of immune response,cellular defense response
323	LKGPGTPAFPH	C/EBP Epsilon	MAPK	0,49	1	0,75	DNA binding protein, Transcription factor	defense response to bacterium,phagocytosis
473	LERQLSLEQEV	Arachidonate 5 lipoxygenase	MAPK2	0,54	1,69	1	Oxidoreductase, Lipid Metabolism - linoleic acid	leukotriene production during acute inflammatory response, inflammatory response, arachidonic acid metabolic process
87	MSTESMIRD	TNF alpha	nd	0,35	1,53	1	Apoptosis, Motility/ polarity/chemotaxis, Cytokine	extracellular matrix organization and biogenesis, activation of MAPK activity, negative regulation of interleukin-6 production
155	SAQGSDVSLTA	HLA-B	nd	0,49	1,55	0,77	membrane protein	antigen processing and presentation, immune response
532	KMAEAYSEIGM	T-cell antigen receptor, zeta	nd	0,38	1,74	1	Receptor	regulation of immune response, cellular defense response
224	GRLRKSLPSLL	Complement component 5 receptor 1	nd	0,5	1,54	1	Receptor, GPCR	defense response to Gram-positive bacterium, activation of MAPK activity, phospholipase C activation, response to peptidoglycan, cellular defense response, immune response, signal transduction, chemotaxis
883	AWTADSGEGDF	Fibrinogen, alpha chain	nd	0,46	1,91	1	Secreted protein	protein polymerization, response to calcium ion
157	VPKRKSLVGTP	PAK5	nd	0,63	1,58	0,83	Ser/Thr kinase	anti-apoptosis
131	SRRPKSSLPPV	Myosin light chain kinase	PAK2	0,72	1,91	1	Ser/Thr kinase	
568	SADSGYIIPLP	PDGF receptor, alpha	PDGFRalpha	0,39	1,72	1	tyrosine kinase	response to cytokine stimulus, positive regulation of cell migration
602	MVRTQTESSTP	PDK1	PDK1	0,56	1,79	1	Ser/Thr kinase	phosphoinositide-mediated signaling, inhibition of apoptosis,response to DNA damage stimulus ,
154	GATMKTFCGTP	AKT1	PDK1	1	1,42	0,77	Ser/Thr kinase	negative regulation of JNK cascade, regulation of nitric-oxide synthase activity, apoptosis, inflammatory response, nitric oxide biosynthetic, G-protein coupled receptor protein signaling pathway
509	ETKGKSFEEIA	Solute carrier family,member 2	PKA	0,39	1,33	1	Membrane protein	cellular lipid metabolic process,
505	PLVQRGSANGL	Beta-adrenergic receptor kinase1	PKA	0,56	1,62	1	Receptor, GPCR	G-protein signaling, adenylate cyclase activating pathway,positive regulation of MAPKKK cascade,endosome to lysosome transport, receptor-mediated endocytosis
210	ELRRMSDEFVD	BCL2 antagonist of cell death	PKA,AKT1	0,42	1	0,7	Apoptosis	phosphoinositide-mediated signaling,induction of apoptosis by extracellular signals,
231	SRKGYSRKGFD	Peptidylglycine alpha amidating monooxygenase	PKC	2,35	1,61	1	Oxidoreductase	peptide metabolic process
766	GLRRSSKFCLK	Beta-2-adrenergic receptor	PKC	0,49	1,6	1	Receptor, GPCR	G-protein signaling, adenylate cyclase activating pathway,positive regulation of MAPKKK cascade,endosome to lysosome transport, receptor-mediated endocytosis
227	IRESESTAGSF	Lck	PKC	1,64	1,96	1	tyrosine kinase	caspase activation,

Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
690	QNLMQSVKETV	Vinculin	PKCalpha	0,37	1,51	1	Adhesion, Cytoskel-etal protein	lamellipodium biogenesis, negative regulation of cell migration
178	KFSKFSLYKQL	PLD1	PKCalpha	0,44	1,48	0,74	Phospholipase	defense response to Gram-positive bacterium, phospholipid catabolic process,small GTPase mediated signal transduction, Ras protein signal transduction, chemotaxis
471	SERRGSHPYID	Phosphodiesterase 7A cAMP-specific high-affinity	Protein_ kinase	0,56	1,94	1	Phosphodiesterase	cAMP-mediated signaling
918	ALSTDSIERLP	PDHK	PDHK	0,38	2,19	1	Protein kinase	small GTPase mediated signal transduction, glucose metabolic process,pyruvate metabolic process
625	EKKAYSFCGTV	Ribosomal S6 kinase	PDHK	0,37	2,73	1	Ser/Thr kinase	stress-activated MAPK cascade,innate immune response
476	GARRSSWRVIS	14-3-3-Eta	SDK1	1	1,68	0,81	Adaptor/scaffold	activation of MAPKK activity, activation of pro-apoptotic gene products, small GTPase mediated signal transduction
822	SSDDDYDDVDI	HPK1	SYK	0,49	1,94	1	Ser/Thr kinase	activation of JNK activity
786	ADENYYKAQTH	SYK	SYK	0,34	1,44	1	tyrosine kinase	activation of JNK activity, regulation of immune response
563	APEDLYKDFLT	VEGF receptor 2	VEGFR	0,39	1,47	1	receptor tyrosine kinase	positive regulation of focal adhesion formation, positive regulation of positive chemotaxis
819	RQGKDYVGAIP	VEGF receptor 2	VEGFR	0,53	1,86	1	receptor tyrosine kinase	

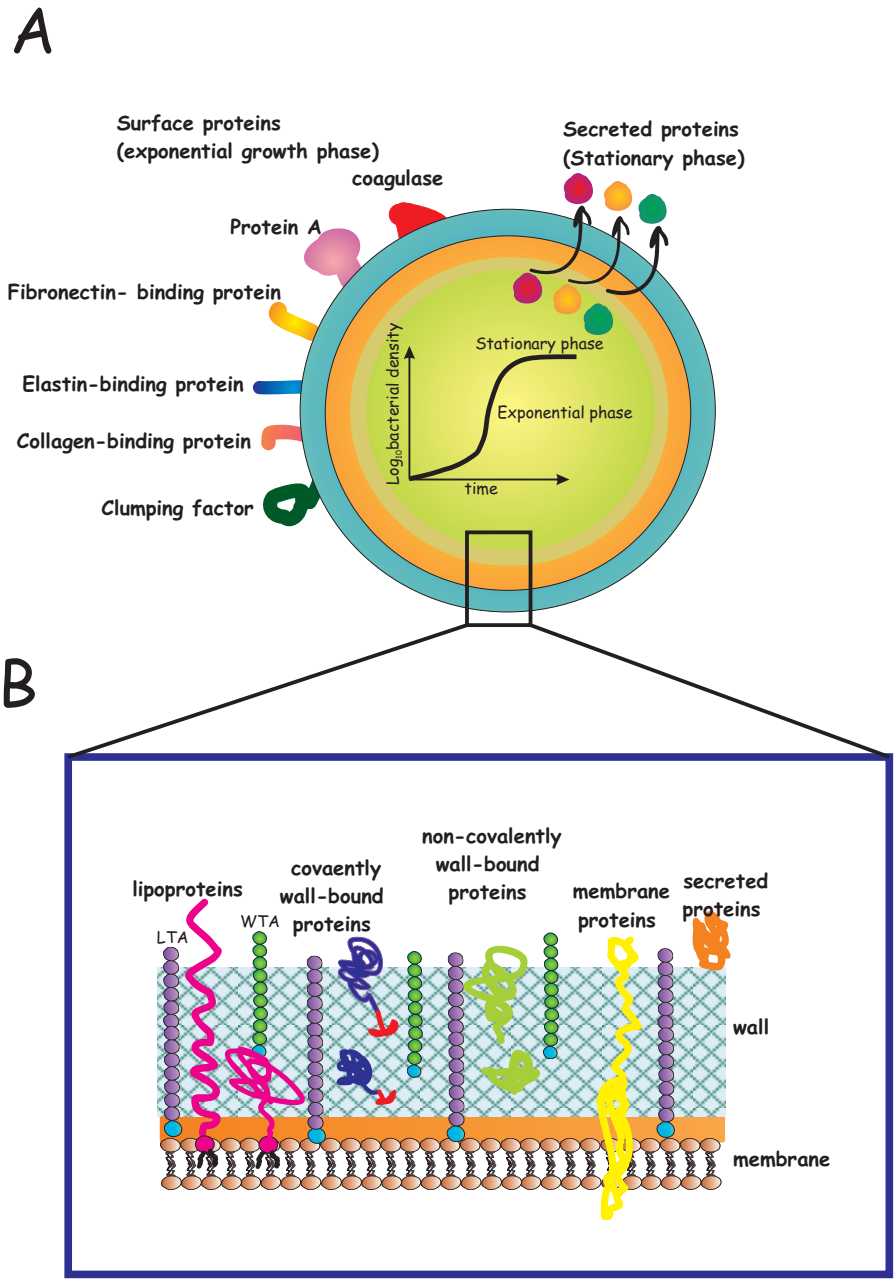
Supplemental Table 3. Kinome profiling of THP-1 macrophages incubated with *S. aureus* NCTC 8325 Δ*pknB*Δ*φ13*

Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
404	EEGEGYEEPDS	CD19	ABL	1,85	1,00	1,00	Membrane protein	regulation of immune response
137	IGEGTYGTVFK	Cyclin dependent kinase 5	ABL	0,35	1,00	1,00	Ser/Thr kinase	activation of pro-apoptotic gene products
873	SEGSSSGRARE	Androgen receptor	AKT	1,80	1,00	0,77	Transcription factor	negative regulation of apoptosis
469	RKRPATDDSST	Cyclin-dependent kinase inhibitor 1B	AKT	0,66	0,75	0,31	Inhibitor protein	DNA damage response,induction of apoptosis
139	TSLAQYDSNSK	Etk	Btk	0,25	1,00	0,64	tyrosine kinase	apoptosis, response to stress
552	ASGYISSLEYP	Complement component 1, subcomponent r	CK1, CK2	0,37	1,00	1,31	Protease	innate immune response
1005	GSDSDSEVDKK	PC4	CK1, CK2	0,38	1,00	0,87	Transcription coactivator	regulation of transcription
684	PSPLPSPTASP	Amphiphysin	cyclin dependent kinase	1,88	1,00	1,00	Vesicle protein	endocytosis
867	PGLRRSPIKKV	B-Myb	cyclin dependent kinase	1,92	1,00	1,00	Transcription factor	regulation of transcription
374	DAPPLSPFPHI	Retinoblastoma like 1	cyclin dependent kinase	0,27	1,00	1,00	Transcription coactivator	regulation of transcription
166	FDGPLSPPLSI	Neurogenic differentiation factor 1	Eph	0,35	1,00	0,88	Transcription factor	positive regulation of apoptosis
192	SDSLSSPTLLA	c-Fos	Eph	0,34	1,00	1,00	Transcription factor	inflammatory response,stress-activated MAPK cascade
166	FDGPLSPPLSI	Neurogenic differentiation factor 1	Eph	0,35	1,00	0,88	Transcription factor	positive regulation of apoptosis
25	NYQVPSPGPSH	T-Cell acute lymphoblastic leukemia 2	ERK	2,00	1,00	0,89	Transcription factor	regulation of transcription
817	PPPQLSPFLQP	Estrogen receptor, alpha	ERK	1,87	1,00	0,59	DNA binding protein	regulation of apoptosis
183	YGGLTSPGLSY	Keratin 8	ERK	0,33	1,00	1,00	Cytoskeletal protein	cytoskeleton organization and biogenesis
192	SDSLSSPTLLA	c-Fos	ERK	0,34	1,00	1,00	Transcription factor	inflammatory response, stress-activated MAPK cascade
399	KTNLSYYEYDK	Etk	FAK	0,25	1,00	1,00	tyrosine kinase	apoptosis, response to stress
269	MTRDIYETDYY	IGF-I receptor	Insulin_ receptor	1,99	1,00	1,00	Receptor tyrosine kinase	PI3kinase pathway, regulation of MAPKKK cascade
449	KAVDGYVKPQI	STAT5A	JAK	1,00	1,86	1,00	Transcription factor	JAK-STAT cascade, cytokine and chemokine mediated signaling pathway
829	LLKLASPELER	c-Jun	JNK	1,85	0,88	1,00	Transcription factor	stress-activated MAPK cascade, innate immune response

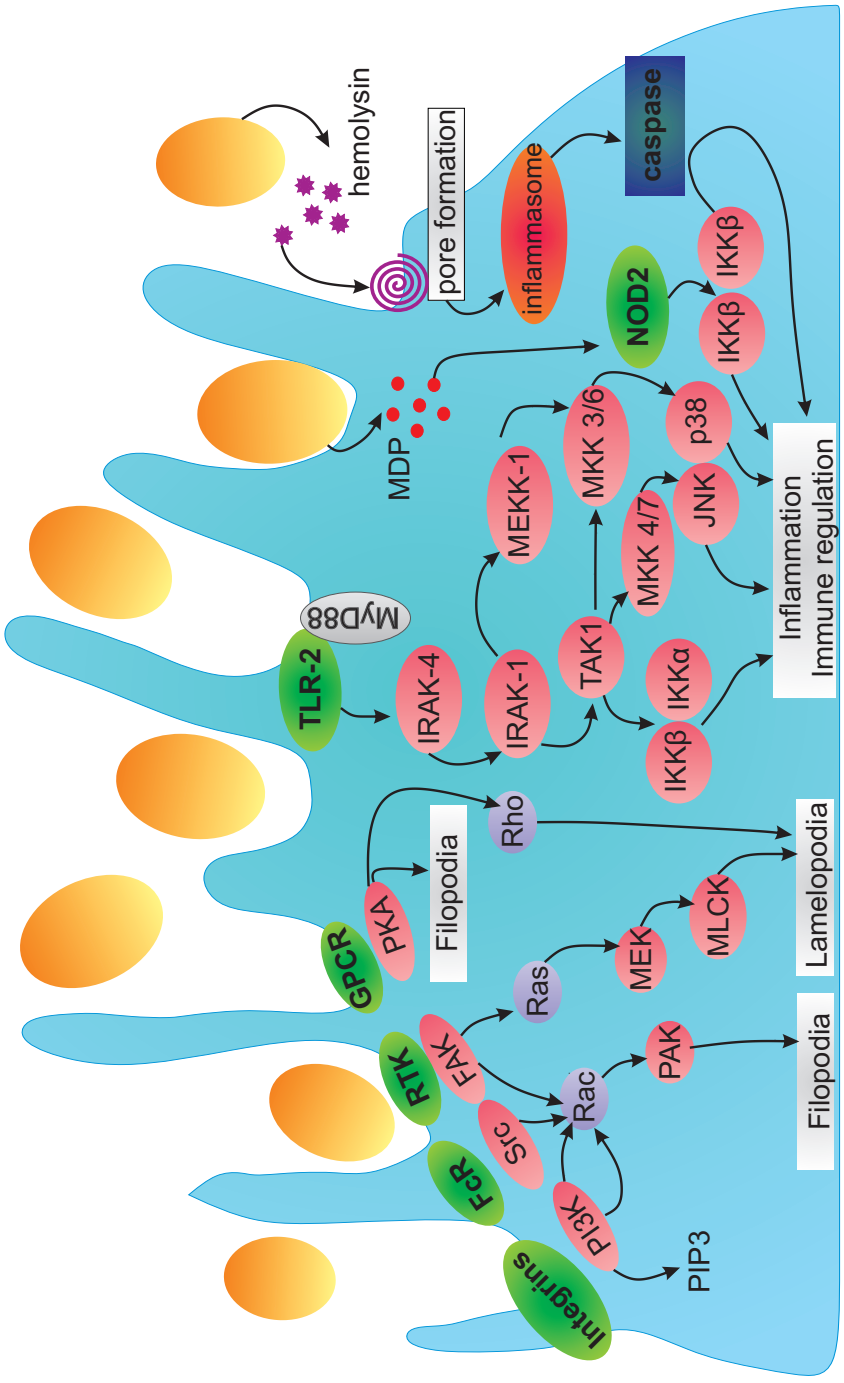
Peptide number	Motif	Protein	Upstream kinase	Fold change			Molecular function	Biological function
				15 min	1 h	24 h		
650	KDGWVYYANHT	WW Domain containing oxidoreductase	JNK	0,28	1,00	1,00	Oxidoreductase	induction of apoptosis
833	DINSLYDVSRM	Phospholipase C, gamma 2	Lyn	1,00	1,81	1,00	Phospholipase	signal transduction, inositol trisphosphate biosynthetic process
773	NTTATYAEPYR	Plakophilin 4	nd	1,99	1,00	1,00	Adhesion	cell adhesion, positive regulation of Rho GTPase activity
760	GSSYGSLMTAH	NPR-B	nd	0,25	1,00	1,00	Lyase,Membrane protein	cGMP biosynthetic process, signal transduction
144	IDAFSDYANFK	Protein tyrosine phosphatase, receptor type, alpha	nd	0,38	1,00	1,00	Receptor protein phosphatase	insulin receptor signaling pathway
760	GSSYGSLMTAH	NPR-B	nd	0,25	1,00	1,00	Lyase,Membrane protein	cGMP biosynthetic process, signal transduction
421	DVDQGSLCTSF	IKK alpha	NIK	0,37	1,00	1,00	Ser/Thr kinase	toll-like receptor 2 signaling pathway, NF-kappaB cascade
293	IKQGEKHSA	Phosphoinositide-3-kinase, catalytic subunit, gamma	PI3K	1,80	1,00	0,82	Autophagy, Carbohydrate Metabolism - inositol phosphate	GPCR signaling, positive regulation of acute inflammatory response
378	QERRGSNVALM	Protein tyrosine phosphatase nonreceptor 7	PKA	1,80	1,00	1,00	Protein phosphatase	nd
62	YQRRASDDGKL	RAF1	PKA	1,80	1,00	1,00	Ser/Thr kinase	apoptosis, MAPKKK cascade, cytoskeleton organization and biogenesis
533	RRRRPTPAMLF	Protein phosphatase 1	PKA	2,42	1,00	1,00	Protein phosphatase	transcription, DNA-dependent
789	SSRRTTLCGTL	Serine/threonine protein kinase 6	PKA	1,84	1,00	0,79	Ser/Thr kinase	cell division, positive regulation of mitosis
1017	RFYPESSYKST	Occludin	PKC	0,27	1,00	1,00	Adhesion, Membrane protein	apoptosis, cell structure disassembly during apoptosis
632	RRGSDSSEDIY	Protein phosphatase 1, regulatory subunit 3A	Ribosomal_S6_kinase_1	0,30	1,00	2,03	Protein osphatase, membrane protein	glycogen metabolic process
11	TATEGQYQQQP	Lyn	Src	2,15	1,00	0,71	tyrosine kinase	negative regulation of MAP kinase activity, response to toxin
834	PKDEVYSKYYT	STAT5B	Src	0,33	1,00	1,00	Transcription factor	JAK-STAT cascade, cytokine and chemokine mediated signaling pathway

Chapter 12

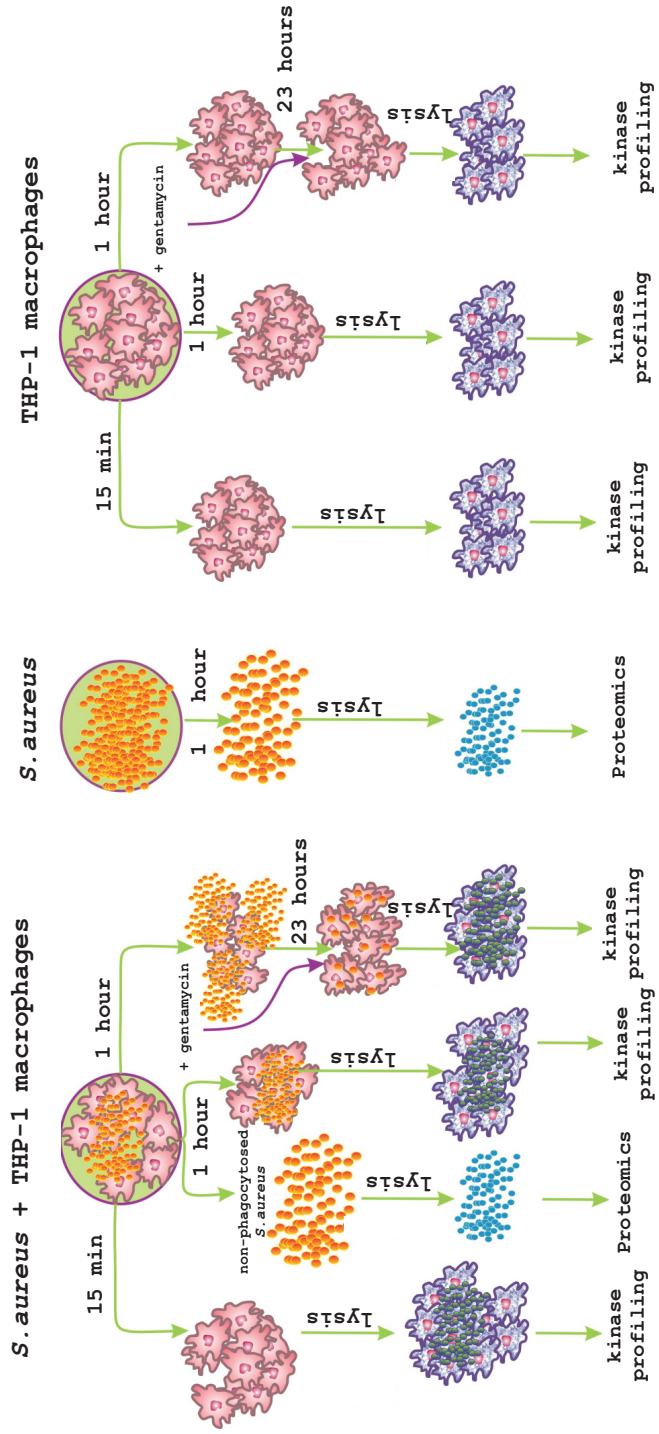
Colour figures



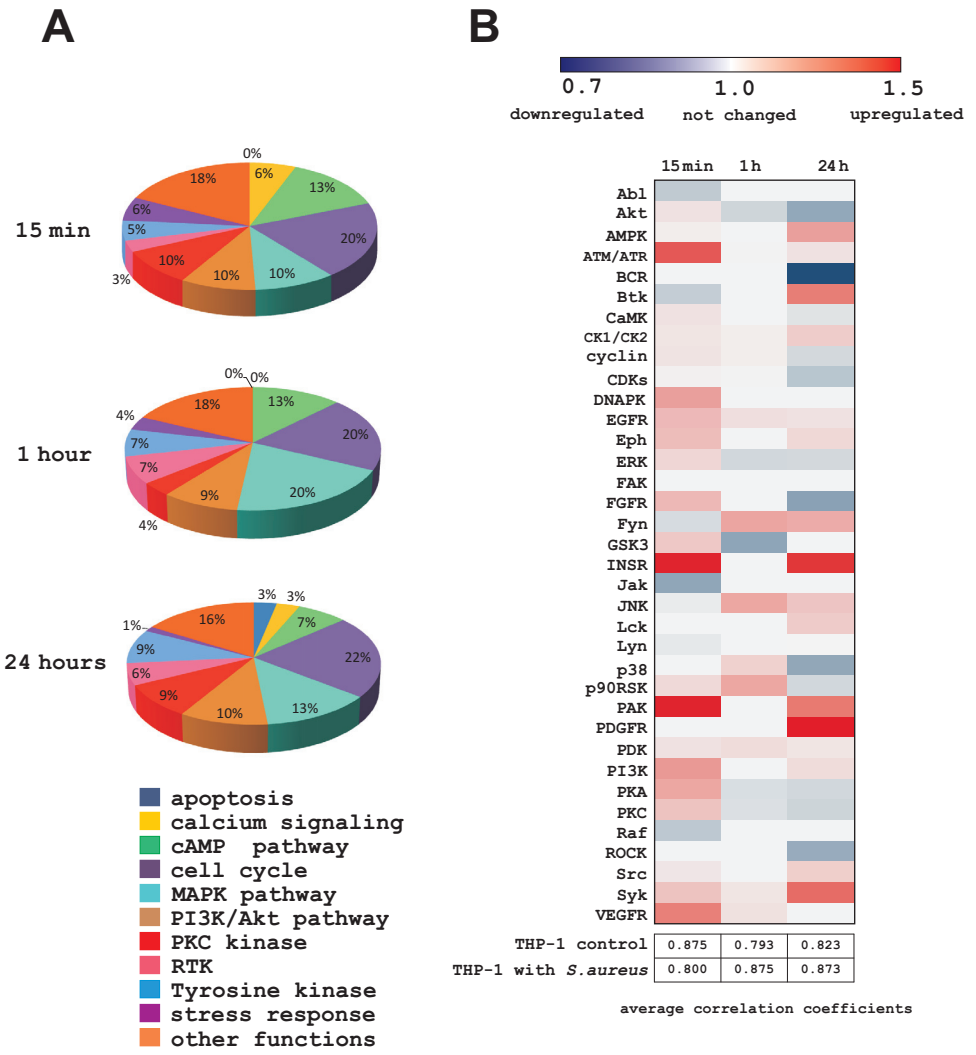
Chapter 1, Figure 3. Schematic representation of staphylococcal virulence factors and their subcellular or extracellular localization.



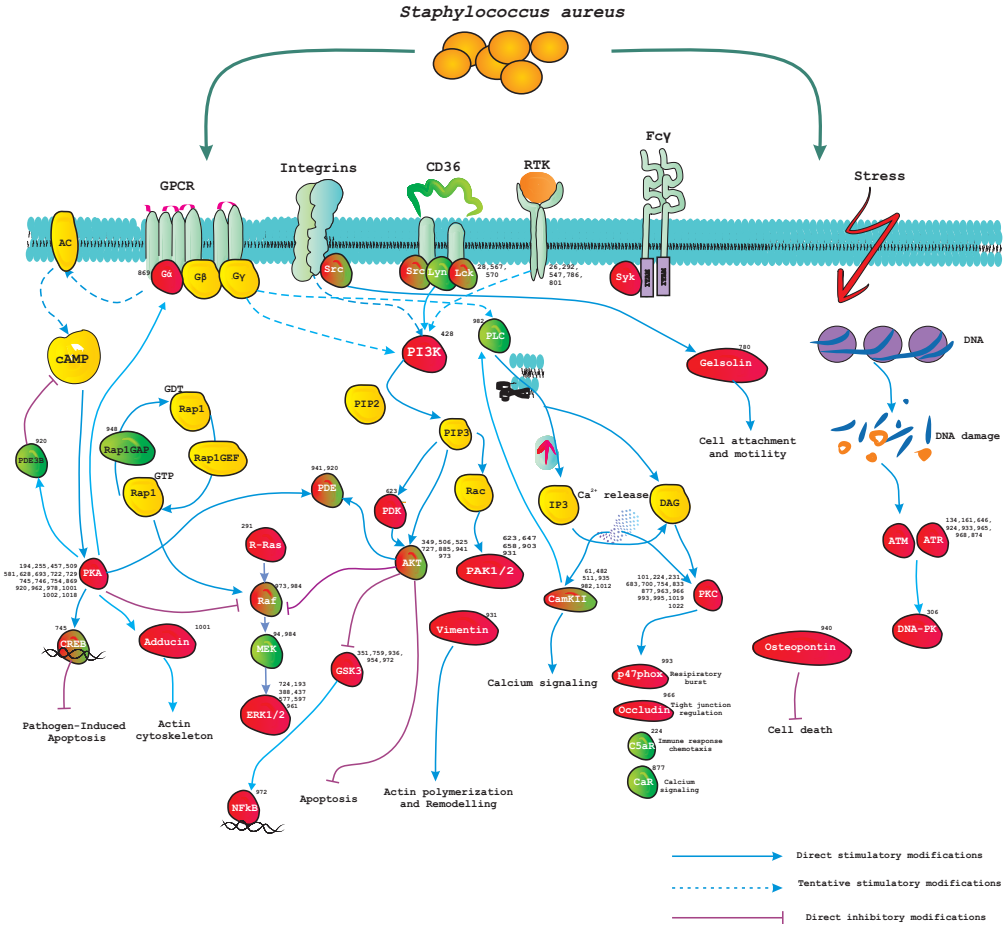
Chapter 1, Figure 4. Signal transduction in macrophages during *S. aureus* infection.



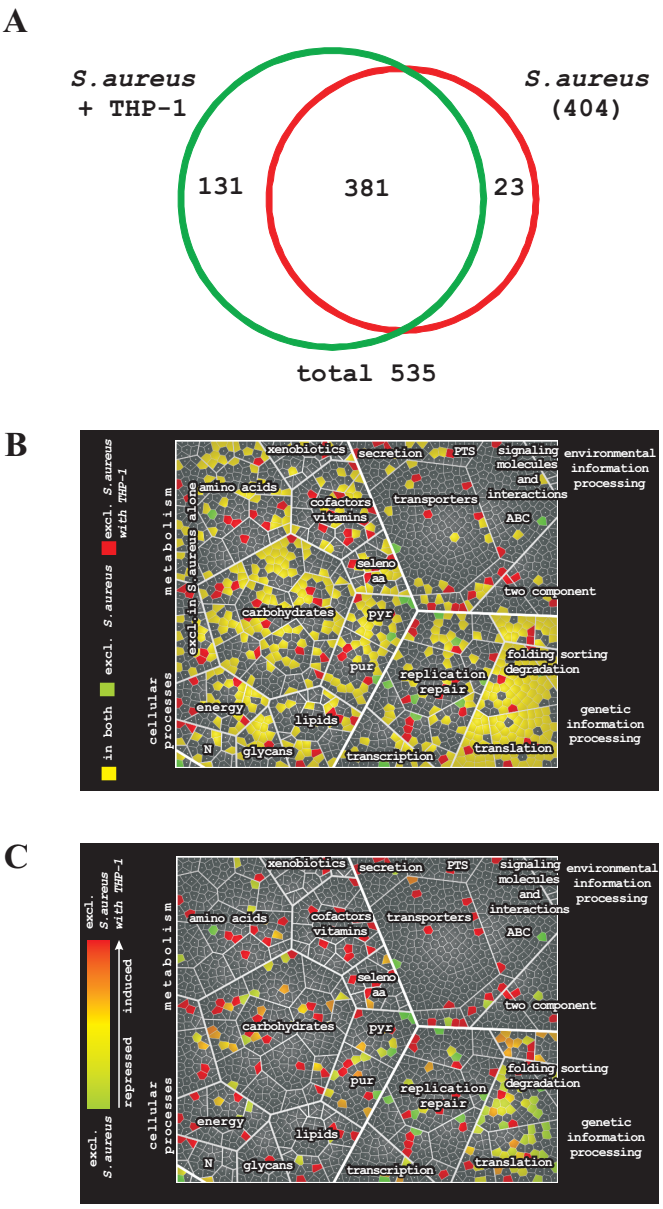
Chapter 2, Figure 1. Schematic representation of work flows. The diagram gives an overview of the work flows to monitor the responses of THP-1 macrophages to *S. aureus* cells by kinase profiling, and (2) the responses of *S. aureus* to THP-1 macrophages by proteomics. For details see Materials and Methods.



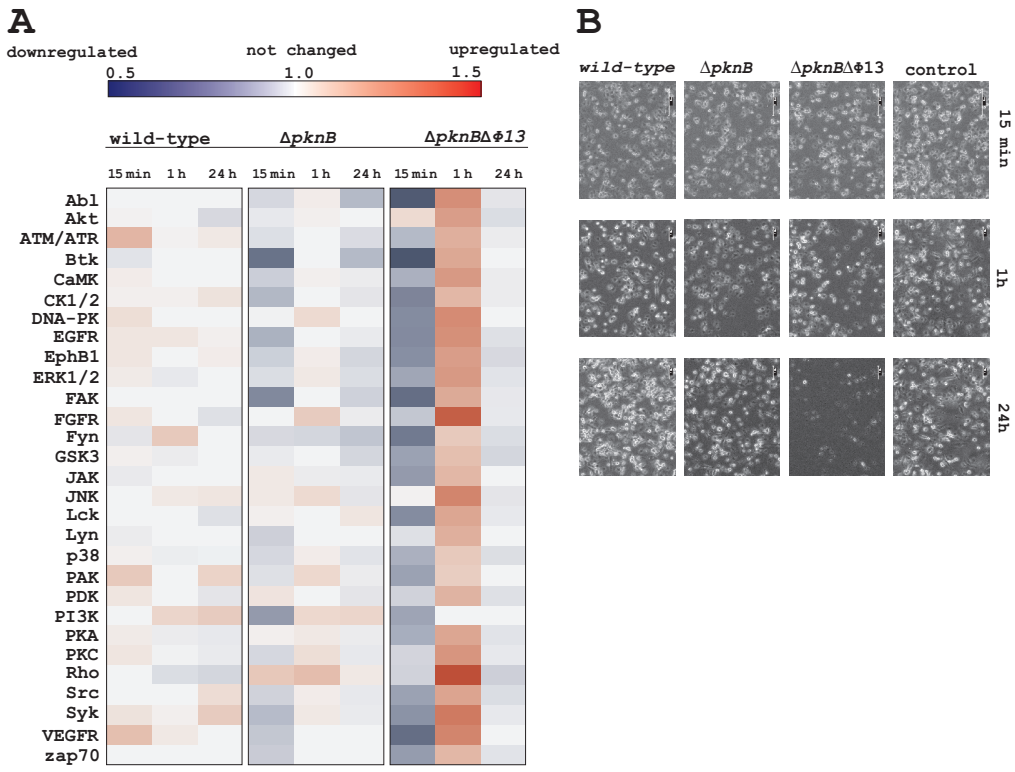
Chapter2, Figure 3. Activity of different kinases and phosphorylation pathways in THP-1 macrophages incubated with *S. aureus*. THP-1 macrophages were incubated with *S. aureus* NCTC 8325 cells for 15 min, 1 h or 24 h. As a control, macrophages were incubated without *S. aureus* cells. Kinase profiling and data analyses were performed as described in the Materials and Methods section. (A) Overview of kinases and signalling pathways that are influenced upon incubation of THP-1 cells with *S. aureus*. (B) Up- and downregulation of kinase activities in THP-1 macrophages incubated with *S. aureus*. A fold change of 1 represents the situation where the phosphorylation of respective peptides is identical in THP-cells incubated with or without *S. aureus*. A fold change of > 1 indicates that the phosphorylation intensity of particular peptides was increased when THP-cells were incubated with *S. aureus*, and a fold change <1 indicates that the phosphorylation intensity of particular peptides was decreased when THP-cells were incubated with *S. aureus*. Calculated values of the 36 presented up- or down-regulated kinase activities are based upon the data in Supplementary Table 3. The Table below the heat map lists the average correlation coefficients of three data sets obtained from samples of THP-1 macrophages incubated with *S. aureus* or the control samples of THP-1 macrophages incubated without *S. aureus* at each time point (15 min, 1 hour and 24 hours). The respective correlation coefficients were derived from the plots presented in Supplementary Figure 3 available on Journal of Proteome Research website http://pubs.acs.org/doi/abs/ 10.1021/pr200224x?prevSearch=miller%2Bmalgorzata&searchHistoryKey=



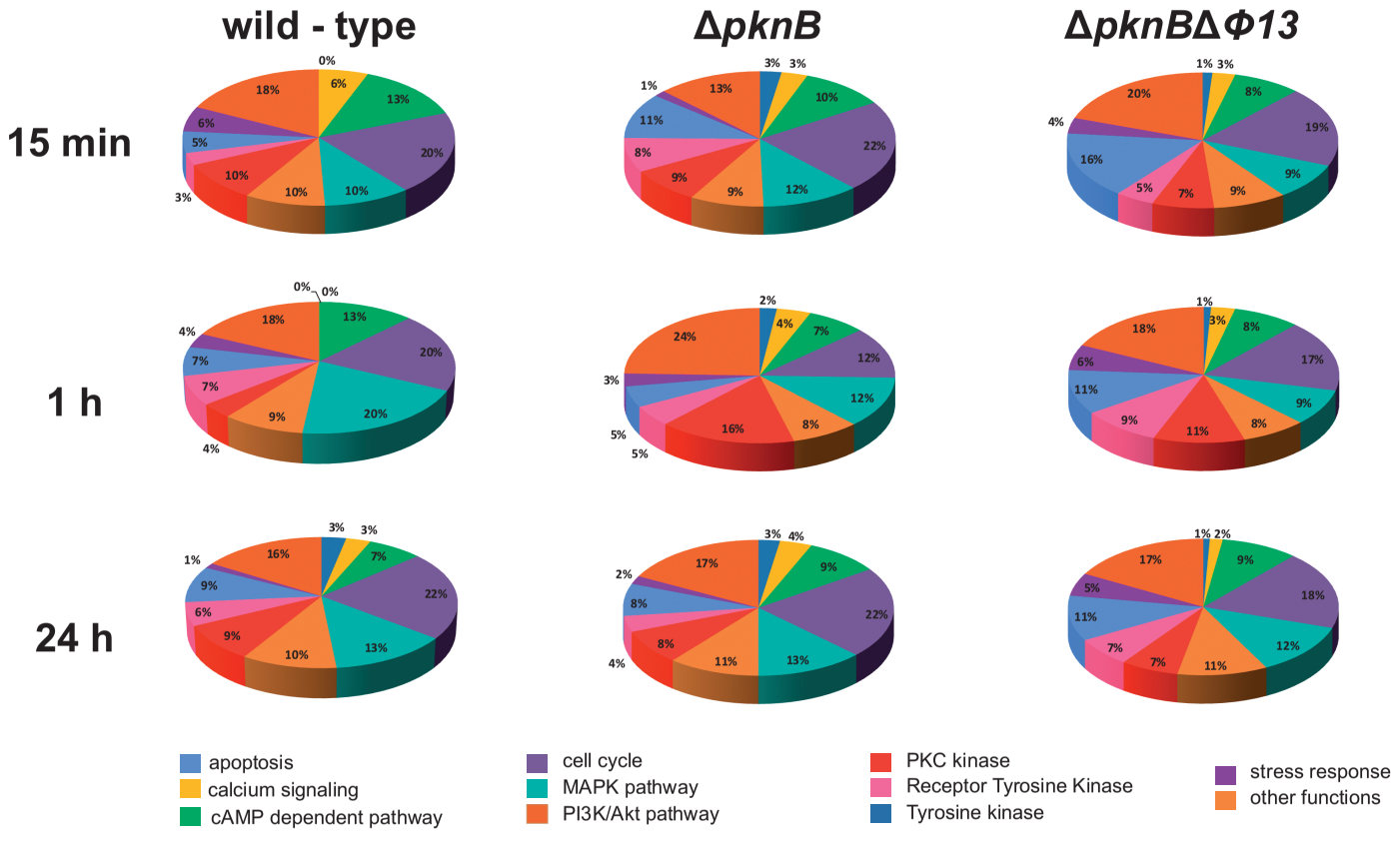
Chapter 2, Figure 4. Overview of the signalling pathways active after 15 min incubation of THP-1 cells with *S. aureus*. The kinase profiling results indicate that during incubation with *S. aureus* three main pathways are active in THP-1 cells, namely the cAMP-dependent pathway, the Ca^{2+}-dependent pathway, and the PI3K pathway. These pathways might be regulated during *S. aureus* infection by different receptors such as the G-protein coupled receptor (GPCR), integrins, the scavenger receptor CD36, the receptor tyrosine kinase (RTK), and the Fcγ receptor. For abbreviations of kinases see Results and Discussion. The numbers indicate the Pepscan numbers of peptides in the arrays that were differentially phosphorylated by extracts of THP-1 cells incubated with or without *S. aureus* (for details see Supplementary Table 1 available on JPR website). Kinases that are downregulated are indicated in green, kinases that are upregulated are indicated in red, and kinases that are possibly involved in the active signalling pathways are indicated in yellow. Direct stimulations are indicated by blue arrows, tentative stimulatory effects are indicated by dashed blue arrows, and inhibitory activities are indicated by red inhibition lines.



Chapter 2, Figure 7. Overview of identified *S. aureus* proteins. (A) The Venn diagram shows the numbers of identified peptides of proteins from *S. aureus* NCTC 8325 that were detected when *S. aureus* was cultivated in the presence or absence of THP-1 macrophages. The Venn diagram was generated using the BioInfoRX Venn diagram plotter (http://bioinforx.com/free/bxarrays/venndiagram.php#). (B) Voronoi treemap based on the KEGG gene orthology for *S. aureus*, visualising the function of the identified proteins. Each small field represents an independent protein. Proteins identified exclusively in *S. aureus* cells cultured in RPMI are indicated in green, proteins found exclusively in *S. aureus* cultured in RPMI in presence of THP-1 macrophages are indicated in red, and proteins present in both conditions are indicated in yellow. Proteins labelled in grey were not detected. (C) Changes in the amounts of *S. aureus* proteins upon incubation with THP-1 cells. Reduced protein levels are indicated in shades of green, and increased levels are indicated in shades of red.

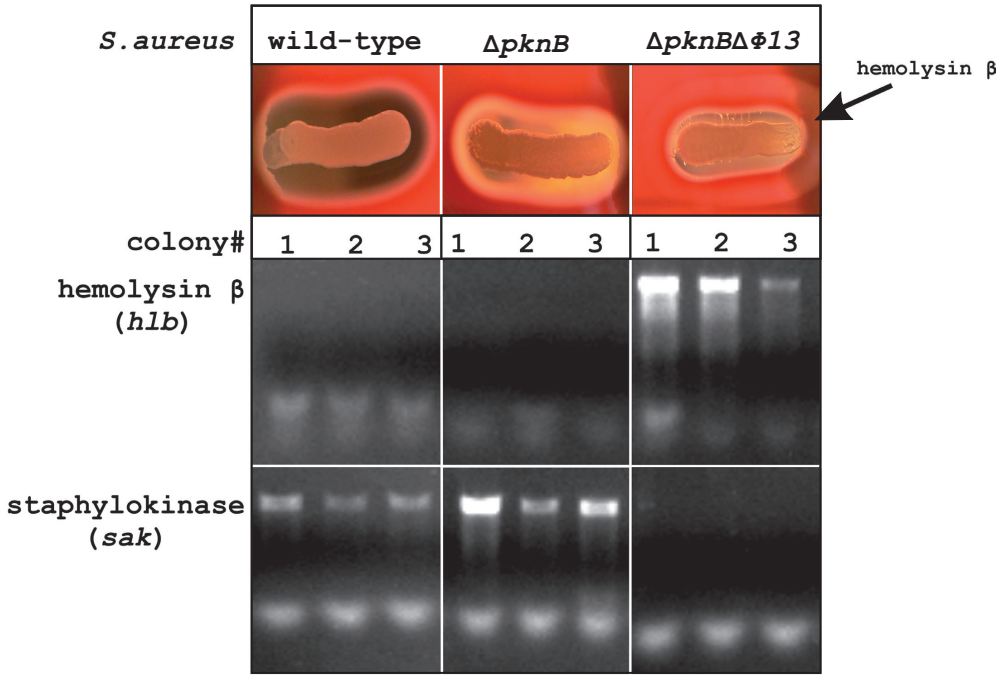


Chapter 4, Figure 3. Activity of different kinases in THP-1 macrophages incubated with pknB mutant *S. aureus* cells. THP-1 macrophages were incubated with cells of the *S. aureus* strains Δ*pknb* or Δ*pknBΔφ13* or the parental strain NCTC 8325 (wild-type) for 15 min, 1 h or 24 h. As a control, macrophages were incubated without *S. aureus* cells. Kinase profiling and data analyses were performed as described in the Materials and Methods section. (A) Up- or down-regulation of kinase activities in THP-1 macrophages incubated with *S. aureus* NCTC 8325, Δ*pknB* or Δ*pknbΔφ13*. A fold change of 1 represents the situation where the phosphorylation of respective peptides is identical in THP-1 cells incubated with or without *S. aureus*. A fold change of > 1 indicates that the phosphorylation intensity of particular peptides was increased when THP-1 cells were incubated with *S. aureus*, and a fold change <1 indicates that the phosphorylation intensity of particular peptides was decreased when THP-1 cells were incubated with *S. aureus*. Calculated values of the presented up- or down-regulated kinase activities are based upon the data in Supplemental Tables 1 and 2. Note that we used the control data set for the parental strain in the previously published paper by [1]. 2011. The parental control data were however collected in parallel with the data sets for the Δ*pknB* or Δ*pknbΔφ13* strains. (B) THP-1 macrophages incubated with *S. aureus* NCTC 8325 (wt), Δ*pknB* or Δ*pknBΔφ13* for 15 min, 1h or 24 h. Magnification 10x.

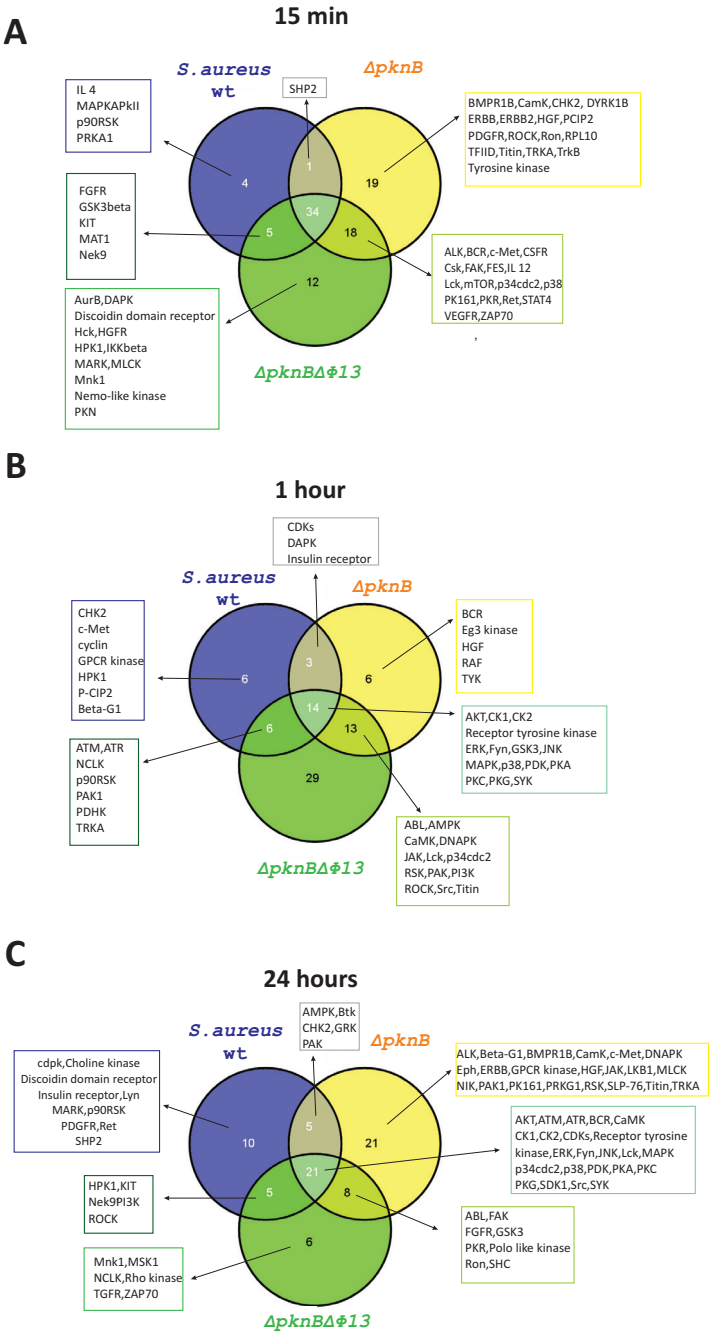


Chapter 4, Figure 4. Overview of kinases and signaling pathways that are influenced upon incubation of THP-1 cells with the *S. aureus* strains NCTC 8325 (wild-type), Δ*pknB* or Δ*pknB*Δ*φ13*. The activity of different kinases and phosphorylation pathways in THP-1 macrophages incubated with *S. aureus* were determined as described in Figure 3. THP-1 macrophages were incubated with *S. aureus* cells for 15 min, 1 h or 24 h. As a control, macrophages were incubated without *S. aureus* cells. Note that we used the control data set for the parental strain in the previously published paper by [1]. 2011. The parental control data were, however, collected in parallel with the data sets for the Δ*pknB* or Δ*pknbΔφ13* strains.

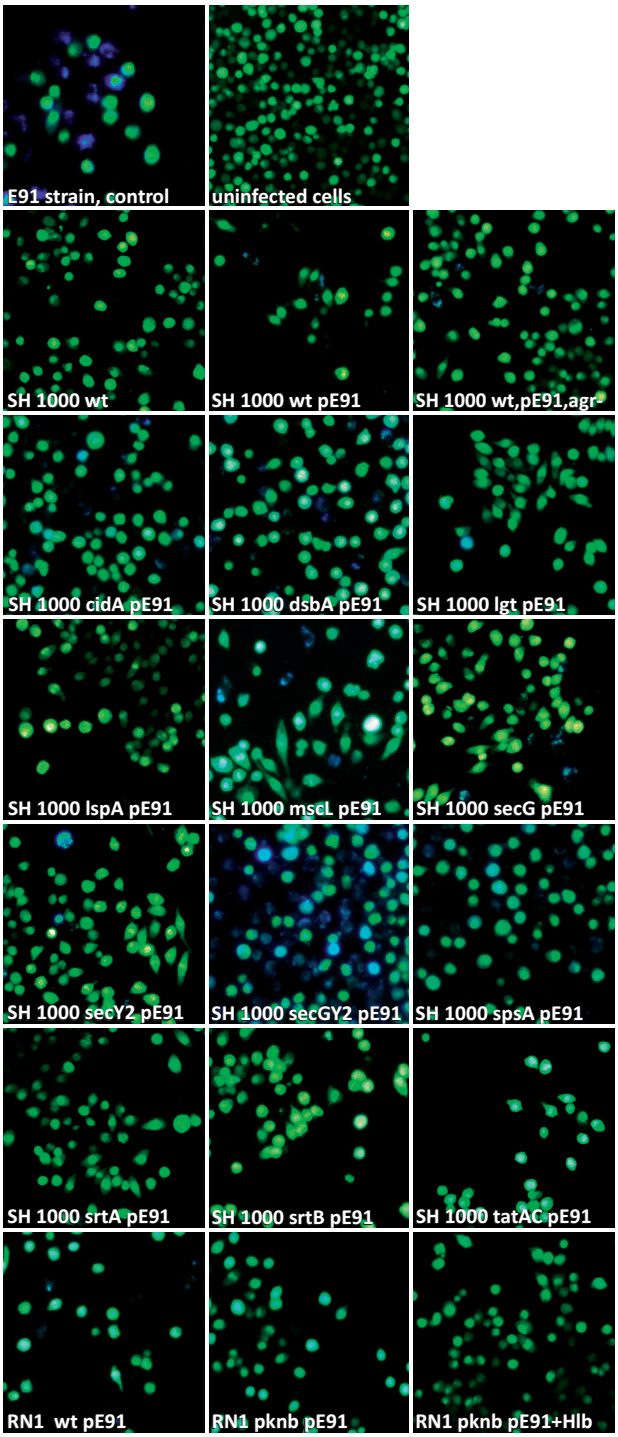
Chapter 4, Figure 5. General overview of the signaling pathways active upon incubation of THP-1 cells with *S. aureus* lacking PknB. The kinase profiling results indicate that during incubation with *S. aureus* Δ*pknB* the activities of several kinases are down-regulated, especially the Focal Adhesion Kinase (FAK), Src kinase, Bruton's kinase (Btk) and phosphatidylinositol-3-kinases (PI3K). Kinases that are down-regulated are indicated in green, kinases that are up-regulated are indicated in red, and kinases that are possibly involved in signaling are indicated in yellow. Direct stimulations are indicated by arrows, and tentative stimulatory effects are indicated by dashed arrows. MDP, muramyl dipeptide; MKK, mitogen-activated protein kinase; PAMP, pathogen-associated molecular patterns. All other abbreviations are defined in the main text.



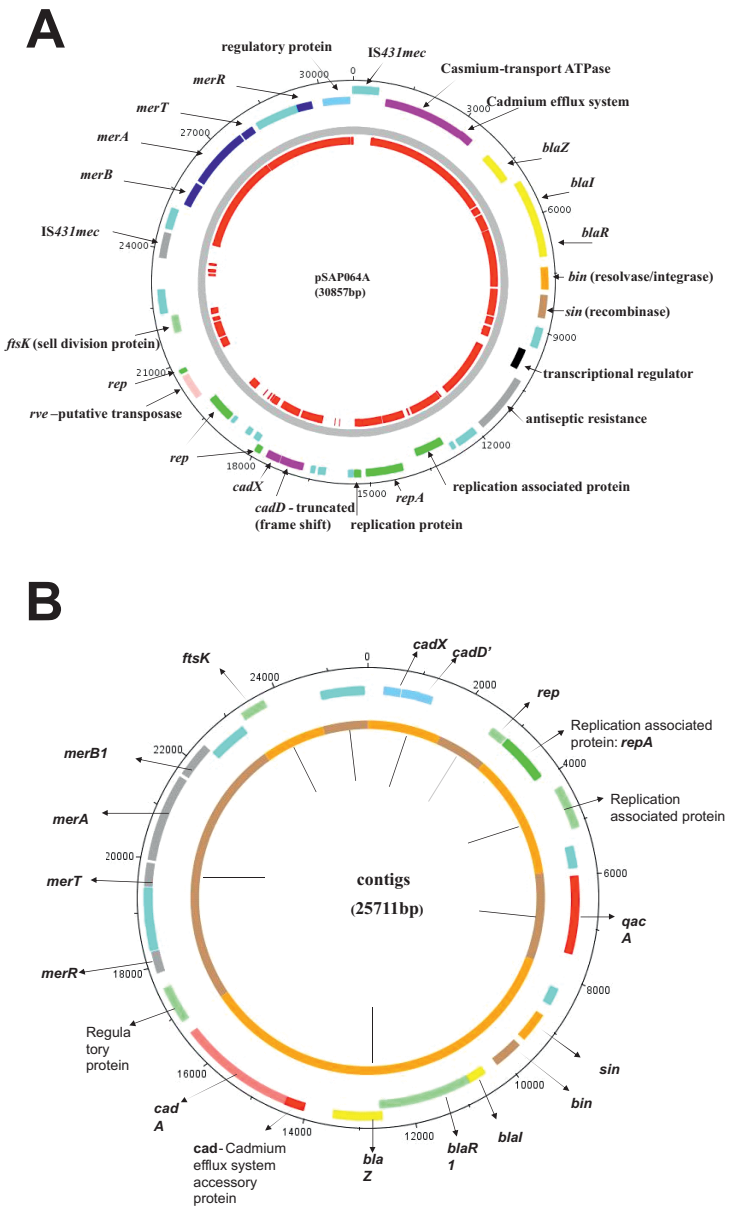
Chapter 4, Figure 7. Production of βhemolysin. Upper panel, Colonies of *S. aureus* NCTC 8325 (wt), Δ*pknB* or Δ*pknB*Δ*φ13* (from left to right) grown on blood agar. Note that the colony of *S. aureus* Δ*pknB*Δ*φ13* forms a faint second halo due to the secretion of active hemolysinβ. Middle panel, PCR detection of the intact hlb gene in three isolates of each strain. Lower panel, PCR detection of the phage 13-encoded sak gene in three isolates of each strain. Strains NCTC 8325 and Δ*pknB* show the typical hlb and sak PCR amplification patterns of strains harboring the lysogenic Hlb-converting phage 13. In contrast, PCR reveals the presence of an intact hlb gene in the phage 13-cured strain Δ*pknB*Δ*φ13*.



Chapter 4, Figure 8. Upstream kinases of which the activity is differentially regulated upon incubation of THP-1 macrophages with or without *S. aureus*. (A) 15 min incubation, (B) 1 h incubation or (C) 24 h incubation. Blue, *S. aureus* NCTC 8325; yellow, *S. aureus* Δ*pknB*; green, *S. aureus* Δ*pknBΔφ13*.



Chapter 5, Figure 1 and Discussion, Figure 2 Secretion of penicillinase into J774 macrophages by different strains of *S. aureus*. The secretion of an active penicillinase by wild-type *S. aureus* or mutant *S. aureus* strains with different protein secretion defects was assessed by fluorescence microscopy. For this purpose, the infected macrophages were loaded with CCF2-AM. Cells in which intracellular secretion of penicillinase has occurred appear blue.



Chapter 5, Figure 4. (A) Schematic circular diagram of the reference plasmid pSAP064A (outer circular diagram). Known CDSs are annotated and colored. Hypothetical proteins are represented by light blue blocks. The inner concentric red circle represents FASTA best matches shared between the reference plasmid and data obtained from plasmid sequencing of strain E91. **(B)** The hypothetical structure of the sequenced plasmid from strain E91 is shown by a schematic circular diagram. The outer circular diagram represents annotated CDSs also marked with different colors. Open reading frames of unknown function are indicated by light blue. The inner circular diagram represents ordered contigs obtained from the assembly.